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Neuroimmune interactions drive chronic itch and inflammation

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Neuroimmune Interactions Drive Chronic Itch and Inflammation

By

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Professor Diana M. Bautista, Chair
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Abstract

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Chronic itch remains a highly prevalent disorder with limited treatment options. Most chronic itch diseases are thought to be driven by both the nervous and immune systems, but the fundamental molecular and cellular interactions that trigger the early development of itch, and the acute-to-chronic itch transition, remain unknown. Here, we show that skin-infiltrating neutrophils are key initiators of itch behaviors in atopic dermatitis, the most prevalent chronic itch disorder. Neutrophil depletion significantly attenuated itch-evoked scratching behaviors in a mouse model of atopic dermatitis. Strikingly, neutrophils were required for several key hallmarks of chronic itch, including upregulation of inflammatory cytokines, skin hyperinnervation, enhanced expression of itch signaling molecules upregulation of activity-induced genes and markers of neuropathic itch in spinal cord and sensory ganglia. We also demonstrate that induction of CXCL10, a ligand for the CXCR3 receptor that promotes acute itch via direct activation of itch sensory neurons, is neutrophil-dependent. Indeed, we find that blockade of CXCR3 signaling attenuates atopic dermatitis itch. Our findings define a new role for neutrophils in atopic dermatitis, and establish the importance of CXCR3 signaling in bridging neutrophils and neurons in the transition from acute to chronic itch.

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Acknowledgements

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Finally, I would like to extend my gratitude to my mentor, Dr. Diana M. Bautista, for her guidance and support throughout my graduate work.

Preface

My primary focus during my dissertation research has been, broadly speaking, the cellular and molecular mechanisms of chronic itch. Within this topic, I have worked specifically on neuroimmune interactions in the context of atopic dermatitis pathogenesis. My colleagues and I found that neutrophils are required to trigger the development of itch during the first eight days of a mouse model of atopic dermatitis (AD), and that these scratching behaviors were further dependent on CXCR3 signaling. Another main finding from this research was the surprising discovery that the TSLPR signaling pathway, long considered canonical in allergic inflammatory diseases such as AD, is not necessary for itch until the second week of the MC903 model despite being required for infiltration of basophils and CD4+ T cells to lesional skin. The timing of the contributions of the many cell types that drive AD disease is poorly understood, and our work makes an important contribution to unraveling this question. Furthermore, the discovery that neutrophils play such a key role in the early stages of AD pathogenesis has implications for the development of novel therapeutics, particularly since many AD patients struggle to control their symptoms with existing treatments. This research comprises the second chapter of my dissertation, and is put into context by the first chapter, a published book chapter that gives an overview of the field of neuroimmune interactions in chronic itch.

In the last chapter of this dissertation, I offer perspectives and thoughts on future directions for my primary thesis work on neuroimmune interactions in atopic dermatitis pathogenesis. The highly collaborative nature of my project resulted in a paper with multiple fascinating avenues of possible further investigation, and I am grateful to have worked with such a talented group of people, each of whom made a unique contribution to the work presented in this dissertation.

Chapter 1: Introduction to itch and neuroimmune interactions driving chronic itch and inflammation.

Previously published as: Walsh CM and Bautista DM. Neuroimmune interactions in chronic itch. November 2018. The role of innate immunity in CNS disorders throughout the lifespan, chapter. (Garden G, Lipton S, Neumaier J, eds). pp. 31 – 42.

Introduction

Chronic itch is a debilitating disorder that affects millions of people worldwide. Chronic itch is driven by cooperative interactions between neurons, epithelial cells and immune cells. However, the molecular mechanisms underlying these interactions are not well understood. Here we review the current understanding of the molecular and cellular mechanisms in the periphery that drive itch disorders and discuss open questions about the neuro-immune interactions that trigger inflammatory itch pathogenesis**.**

Acute Itch

Acute itch is an extremely common experience: it can be caused by an insect bite, contact with an irritating plant such as poison ivy, or even a wool sweater. While unpleasant, the sensation of itch, like pain, is thought to play a fundamental role in survival by teaching us to avoid irritating elements in our environment. Itch triggers the protective reflex of scratching that promotes removal of irritating plants as well as insects and helminths capable of transmitting diseases such as zika or malaria. In contrast to acute itch, chronic itch is a profoundly debilitating condition that affects roughly 10% of the adult population(Wilson et al., 2013). Chronic itch remains poorly understood and difficult to treat, making it a tremendous clinical problem.

Itch is mediated by a subset of somatosensory neurons, a highly heterogeneous group of cells that transduce touch, itch, and pain sensations. Somatosensory neurons have their cell bodies in the trigeminal and dorsal root ganglia (DRG) and each has a single bifurcated axon with one branch innervating the skin and the other innervating the dorsal horn of the spinal cord(Carstens et al., 2014). The peripheral afferents that innervate the skin transduce environmental stimuli into electrical signals that travel to the central afferent of the neuron and synapse onto a variety of second-order interneurons in the spinal cord (Figure 1).

Projection neurons in the spinothalamic tract project from the spinal cord to the parabrachial nucleus and the thalamus and activate 3rd order neurons that project to the somatosensory cortex. While itch signaling in the central nervous system remains very poorly understood, fMRI data reveals that itchy stimuli activate both unique areas and some of the pain regions of the primary somatosensory cortex, as well as a myriad of brain areas involved in emotional processing, reward, evaluation, and motor control(Dong and Dong, 2018).

Cells and Molecules of Acute Itch

Studies have demonstrated two broad classes of itch neurons (termed pruriceptors): histamine-dependent and histamine-independent. While histamine signaling contributes to many forms of acute and allergic itch, chronic itch tends to be dominated by non-histaminergic mechanisms and therefore does not respond to antihistamines. An astonishing array of pruritogens leads to activation of distinct subsets of pruriceptors, including factors derived from keratinocytes, a wide variety of immune cells, and exogenous triggers (Table I).

Recent studies have identified TRPA1 and TRPV1 as two of the primary ion channels responsible for driving acute itch excitability. Activation of these channels promotes the opening of voltage-gated ion channels that trigger action potential firing and synaptic transmission to activate spinal neurons in the dorsal horn. Several molecules have been shown to play a key role in itch transmission within the spinal cord including glutamate, gastrin-releasing peptide (GRP), natriuretic peptide b (NPPB) and dynorphin(Dong and Dong, 2018; Hoon, 2015; Kardon et al., 2014). Glutamate is believed to have a specific role in pain signaling and may inhibit itch: genetic deletion of the glutamate transporter, VGLUT2, from nociceptive neurons was found to enhance itch behaviors (Dong and Dong, 2018). DRG neurons transmit itch signals to second-order spinal cord neurons via neuropeptides, including NPPB. $NPPB^{+}$ DRG neurons synapse onto neurons which express the NPPB receptor, NPRA, as well as the neuropeptide GRP. These neurons then release GRP onto a group of interneurons in the dorsal horn that express the GRP receptor, GRPR(Dong and Dong, 2018). Conversely, some groups have found evidence that DRG neurons are able to release GRP directly onto GRPR+ spinal interneurons. Itch signaling in the spinal cord is highly complex and is an area of ongoing debate and investigation (Figure 2).

Chronic Itch

While many of the molecules and neurons that drive acute itch have been elucidated in the last decade, the molecular and cellular mechanisms that trigger chronic itch remain enigmatic. Chronic itch arises from dysfunction of neurons, immune cells, and epithelial cells at multiple sites, including the periphery, spinal cord and brain. Psoriasis and atopic dermatitis are two of the most prevalent chronic itch disorders. Neuronal and immune dysregulation lead to the distinct clinical phenotypes observed in these disorders. As such, psoriasis and atopic dermatitis have been the target of contemporary studies aimed at defining molecular and cellular mechanisms that contribute to disease phenotypes.

Atopic dermatitis is an allergic inflammatory disorder that typically begins in childhood. It affects roughly 10-20% of the population worldwide(Bantz et al., 2014). AD is characterized by skin barrier dysfunction, thickened lesions and extreme chronic itch(Spergel and Paller, 2003). It is believed to be caused by a complex interplay between genetic and environmental factors, and is often the first step in a process known as the atopic march: children with atopic dermatitis are significantly more likely to

develop asthma and allergic rhinitis(Spergel and Paller, 2003). Atopic dermatitis is difficult to treat and causes tremendous psychological and financial burdens for the patients and families affected. There is an urgent need for a better understanding of the pathology of this disorder as well as improved therapies.

Psoriasis is a chronic inflammatory skin disease characterized by defects in keratinocyte differentiation, epidermal thickening, and raised scaly plaques on the skin. Severe itching is a hallmark symptom of this disorder. It affects 2-3% of the population and is considered an “immune centric” disorder, due to the fact that disease clearance is observed after immunosuppressive therapies(Golden et al., 2013). The T cell derived cytokines IL-17 and IL-23 are believed to be particularly important, and biologic therapies that block these cytokines have recently been developed with excellent results in patients(Guttman-Yassky and Krueger, 2017).

Until recently, itch research has mainly focused on the roles of epithelial cells and immune cells in driving itch and inflammation. However, contrary to the long-held viewpoint in which immune cells release mediators that trigger activation in somatosensory neurons, leading to chronic itch, a number of contemporary studies have demonstrated that both short and long term signaling processes go both ways between neurons and immune cells in the periphery. Furthermore, several receptors canonically believed to be expressed only in immune cells or neurons have been found to play key roles in multiple cell types. These findings demonstrate that an ongoing conversation between immune cells and somatosensory neurons is required to drive itch and inflammation in the atopic dermatitis disease state. Future therapies will depend on furthering our understanding of this conversation.

Immune Cells Modulate Neuronal Function in Chronic Itch

The direct activation of neurons by immune cells in the context of atopic dermatitis remains an important avenue whereby itch and inflammation are induced. In addition to simply activating neurons, immune cells change the signaling behavior of somatosensory neurons in a number of complex ways, including lowering excitation thresholds, evoking neurite outgrowth, and facilitating immune cell-neuron contacts in the periphery. Four immune cell types have been shown to directly influence neuronal excitability, sensitization and branching in the context of chronic itch: Langerhans cells, T cells, eosinophils and mast cells (Figure 3). A consistent theme in the story of immune cell signaling to neurons is the neuronal expression of receptors long believed to function only in immune cells, including, a variety of canonical immune cell receptors, e.g. IL-31Rα, IL-4Rα, IL-13Rα1 (Table I), among others, are expressed and active on somatosensory neurons, as described below.

Langerhans cells are dendritic cells located in the epidermis and are important in initiating cutaneous immune responses. Their dendritic processes are located in the stratum corneum, the most superficial layer of the skin, and promote skin barrier homeostasis(Jaitley and Saraswathi, 2012; Seneschal et al., 2012; Yoshida et al.,

2014). Like other types of dendritic cells, Langerhans cells respond to antigens from the external and internal environment and upon activation, influence T cell activation and migration(Ding et al., 2008). A role for Langerhans cells in atopic dermatitis comes from both human and animal studies. Human atopic skin contains significantly higher numbers of Langerhans cells and they display an altered morphology, with more dendritic branches that extend through tight junctions towards the superficial epidermis. These branches have the molecules that capture exogenous antigens, including haptens or viral antigens. These antigens are presented to T cells, triggering activation(Dubrac et al., 2010; Nakajima et al., 2012). Cultured murine Langerhans cells produce nitric oxide in response to the potent inflammatory agents lipopolysaccaride and interferon-gamma(Qureshi et al., 1996). Nitric oxide is a well-known agonist of the TRPA1 and TRPV1 ion channels, which have been shown to play key roles in many forms of acute and chronic itch(Bautista et al., 2014; Kittaka and Tominaga, 2017). Furthermore, one study(Morita et al., 1995) showed decreases in pruritis among atopic dermatitis patients in response to a topical nitric oxide synthase inhibitor, suggesting a role for this molecule in AD pathogenesis. More direct evidence for a role of Langerhans cells comes from mouse models of AD, in which depletion of Langerhans cells significantly decreases skin inflammation and lesions(Elentner et al., 2009).

T cells play a key role in atopic dermatitis. While T cells have long been known to communicate with other immune cells and epithelial cells, their interactions with somatosensory neurons under chronic itch conditions were unknown until recently. There are multiple kinds of T cells, including cytotoxic T cells, which kill cells that are infected with bacteria or viruses, and helper T cells, which secrete cytokines that then act on other types of cells. Helper T cells are further specialized into subtypes, such as Th1, Th2, Th17, etc., depending on their mode of activation (e.g., viral or bacterial infection, allergen exposure, etc.). The Th17 subtype of T cells is a key mediator of psoriasis(Guttman-Yassky and Krueger, 2017). Th17 cells secrete a variety of inflammatory interleukins which in turn alter the activity of many genes involved in skin barrier function that trigger psoriatic phenotype. In contrast, the Th2 subtype of T cells is activated in essentially all atopic dermatitis patients, while Th17 and Th1 activation is additionally seen only in a subset of patients(Guttman-Yassky and Krueger, 2017). Cyclosporine is a potent immunosuppressant that acts to inhibit T-cells by inhibiting signal transduction downstream of T Cell Receptor activation. Cyclosporine very effectively suppresses the atopic dermatitis disease phenotype short-term(Khattri et al., 2014), but due to relapse and renal toxicity, is not an effective long-term therapy.

IL-31, a cytokine largely derived from Th2 cells, is highly upregulated in atopic dermatitis patients(Nobbe et al., 2012). Cevikbas and colleagues discovered that IL-31 evokes calcium transients in cultured DRG neurons and triggers itch in mice upon injection into the skin(Cevikbas et al., 2014). These results were dependent upon the ion channels TRPV1 and TRPA1, which play key roles in immune cell function. Moreover, IL-31 has been implicated in neurite outgrowth(Feld et al., 2016). Increases in nerve fiber density are observed in the skin of AD patients, likely contributing to their

itch and inflammation symptoms(Tominaga and Takamori, 2014). Indeed, overexpression of IL-31 in lymphocytes or intradermal injection of the protein in mice triggers dramatic sprouting of sensory nerve fibers in the skin(Feld et al., 2016).

In addition to Th2 cells, eosinophils are also thought to be key regulators of sensory neuron innervation in chronic itch. Eosinophils are a type of white blood cell granulocyte that mediate viral, bacterial and fungal defense and immunoregulation(Radonjic-Hösli and Simon, 2014; Simon et al., 2004). In atopic dermatitis patients, eosinophil counts and eosinophil granule proteins are elevated in peripheral blood, and correlate with disease severity(Simon et al., 2004). The number of eosinophils is also increased in the skin in multiple chronic itch mouse models(Foster et al., 2011; Li et al., 2006). A recent study demonstrated that eosinophils contribute to skin barrier dysfunction in a mouse model of atopic dermatitis(Naidoo et al., 2018), underscoring the importance of these cells in AD pathology. Eosinophils have been recently demonstrated to increase somatosensory neurite branching in mouse neuron-eosinophil co-cultures, and when sensory neurons were treated with medium from these co-cultures. These results suggest that the close functional interaction between eosinophils and neurons is necessary for full eosinophil-mediated increase in neurite branching(Foster et al., 2011). Whether such interactions drive hyperinnervation in atopic dermatitis *in vivo* has yet to be directly examined. While psoriatic lesions also display hyperinnervation(Taneda et al., 2011), it is unclear whether eosinophils and/or T cells contribute to this phenotype.

Mast cells are tissue-resident granulocytes that contribute to inflammatory chronic itch via degranulation and release of inflammatory cytokines. In human atopic dermatitis, mast cells are more abundant in lesional skin and display a high prevalence of degranulation(Liu et al., 2011). A number of studies have shown that mast cells can both directly activate and sensitize somatosensory neurons in the skin. The products of mast cell degranulation, including serotonin, histamine, Bam8-22, and proteases, potently and directly activate their cognate receptors on somatosensory neurons to drive itch behaviors and to promote neurogenic inflammation via the release of inflammatory peptides. Mast cell-neuron interactions have been shown to be strengthened in a mouse model of atopic dermatitis. Hagiyama et. al. demonstrated that mast cells upregulate expression of intercellular adhesion molecule 1 (ICAM-1), which mediates mast cell-sensory neuron interactions(Hagiyama et al., 2013). These data suggest that the strengthening of mast-cell neuronal interactions may contribute to the increased degranulation and neuronal activation observed in atopic dermatitis.

Mast cells may also regulate the expression of excitatory signaling molecules within sensory neurons, thereby promoting hyperexcitability. Expression of the ion channel TRPA1 is markedly increased in skin biopsies from atopic dermatitis patients as compared to normal skin, and also in the IL-13 mouse model of atopic dermatitis(Oh et al., 2013). TRPA1 is also required for itch behaviors in multiple mouse models of atopic dermatitis(Morita et al., 2015; Oh et al., 2013). Interestingly, mast cell deficient mice do not display the increased expression of TRPA1 in lesional skin. IL-13 also directly

activates and sensitizes DRG neurons and increases itch-evoked scratching behaviors in mice following injection of a variety of pruritogens(Oetjen et al., 2017). This cytokine is released by both Th2 cells and mast cells, though the relative contributions of mast cells vs Th2 derived IL-13 to neuronal excitation is unknown.

Neurons Recruit and Activate Immune Cells in Chronic Itch

Far from being passive recipients of messages from immune cells, neurons actively participate in driving inflammation in a variety of chronic itch disorders (Figure 4). Psoriasis provides an excellent example of this phenomenon: it has long been understood as an "immune-centric" disease, driven by the action of Th17 helper cells. However, multiple lines of evidence support the requirement for neuronal signaling in this disorder, highlighting the complex, multicellular nature of the disease.

Psoriatic plaques have a high expression of neuropeptides and are hyper-innervated by somatosensory neurons(Ostrowski et al., 2011). Clinically, spontaneous remission of psoriasis plaques have been reported in patients following nerve injury, and two pilot studies have demonstrated that injections of botulinum toxin have beneficial effects in psoriasis patients(Campanati et al., 2017; Ward et al., 2012). These clinical observations were recently recapitulated in the lab: treatment with botulinum toxin ameliorated psoriasis-like symptoms in the KC-Tie2 genetic mouse model of psoriasis(Ward et al., 2012), which displays the immunological, epithelial and neural characteristics of human disease. Surgical cutaneous denervation in this model results in significant reductions in epidermal thickening and T cell infiltrate. These deficits could be rescued by intradermal injection of calcitonin gene related peptide (CGRP), a potent vasodilatory and inflammatory neuropeptide produced by somatosensory nerves or by activating the receptor for substance P (SP), another inflammatory neuropeptide, with a selective agonist(Ostrowski et al., 2011). These data suggest that in addition to T cells, neuropeptide signaling from sensory neurons drives epithelial cell dysfunction and inflammation in psoriasis.

Moreover, recent studies have begun to explore the role played by somatosensory neurons in atopic dermatitis pathogenesis. Neurons signal to and regulate multiple types of immune cells in AD. Indeed, inflammatory neuropeptide release by somatosensory neurons has been shown to dramatically influence Langerhans cell activity. Langerhans cells play a key role in driving the Th2 response in AD. In a series of *in vitro* experiments, Ding et. al. demonstrated that treating cultured Langerhans cells from mouse epidermis with CGRP upregulated their expression of Th2 cytokines. Furthermore, CD4+ T cells to which these CGRP-treated Langerhans cells presented antigens demonstrated Th2-type polarization(Ding et al., 2008). This work indicates that somatosensory neuronal signaling actually promotes a Th2 response, which is central to the pathology of AD.

Eosinophils, noted above for their remarkable ability to evoke neurite outgrowth, also respond to neuronal signaling. Cultured DRG neurons produce the potent and specific

eosinophil chemokine, eotaxin-1, as well as the adhesion molecules ICAM-1 and VCAM-1(Foster et al., 2011). ICAM-1 functions as a ligand for LFA-1, while VCAM-1 binds to VLA-4. Both VLA-4 and LFA-1 are expressed by eosinophils(Lima et al., 2007). Moreover, eotaxin levels are elevated in atopic dermatitis lesions(Owczarek et al., 2010). These data suggest that under atopic dermatitis conditions, neurons call eosinophils to lesions and eosinophils promote additional neuronal sprouting. Clearly, understanding neuron-eosinophil bidirectional signaling is fundamental to increasing our knowledge of these enigmatic cells.

Somatosensory nerve endings have been shown to be in close proximity to mast cells in both chronic itch and pain. Previous work has established that neuron-mast cell interactions are required for pain hypersensitivity(Chatterjea and Martinov, 2015). Somatosensory neurons secrete the metalloprotease MT5-MMP which can trigger mast cell degranulation and regulate neuron-mast cell contact via N-cadherin(Folgueras et al., 2009). Mice lacking this protease display alterations in chronic pain(Folgueras et al., 2009), but chronic itch has not yet been examined in these animals. Within the field of chronic itch, neuronal-mast cell interactions are proving to be equally crucial.

Substance P is an inflammatory neuropeptide released by somatosensory neurons implicated in both chronic itch and pain(Azimi et al., 2017; Chiu et al., 2012). Substance P acts as a robust pruritogen in both humans and rodents. Interestingly, inhibition of the canonical substance P receptor, neurokinin-1 receptor (NK-1R), has no effect on substance P evoked scratching(Azimi et al., 2017). Recent studies have identified a member of the Mas-related g-protein coupled receptor (Mrgpr) family, MrgprB2, as a new target for substance P. Indeed, substance P-evoked itch behaviors are inhibited by MrgprB2 antagonists in both wild-type and *NK-1R* knockout animals(Azimi et al., 2016).

While the Mrgpr family is well-known for mediating neuronal responses to pruritogens(Liu and Dong, 2015), MrgprB2 is exclusively expressed on mast cells. Recent studies have shown that MrgprB2 is required for mast cell activation by a variety of secretagogues, including substance P(McNeil et al., 2014). In fact, substance P does not evoke degranulation in cells transfected with missense mutants in the human orthologue of MrgprB2, MRGPRX2(Alkanfari et al., 2018). These finding highlight a key role for neurons in regulating mast cell function.

Beyond the Periphery

While interactions between neurons and immune cells in the skin are clearly fundamental to the development of chronic itch and pain, neuroimmune signaling in the CNS also plays a key role(Romero-Sandoval et al., 2008). Indeed, persistent activation of microglia in the CNS is believed to drive the persistence of chronic pain, suggesting that glial cells in the CNS may also contribute to chronic itch pathogenesis(Hulsebosch, 2012). While microglia have not been examined in atopic dermatitis or psoriasis mouse models, they may play a key role in a contact hypersensitivity model of chronic itch,

DNFB (Zhang et al., 2015). This is sure to be an active area of research moving forward.

Another recent study focused on the contribution of reactive astrogliosis, a change in the morphology of spinal astrocytes resulting in increased arborization and enlarged cell bodies, to chronic itch. Astrogliosis and expression of the astrocytic marker GFAP was recently demonstrated in the dorsal spinal cord in a mouse model of atopic dermatitis(Shiratori-Hayashi et al., 2015). This was dependent on TRPV1-expressing peripheral itch fibers that innervate the dorsal horn, and observed in areas of the spinal cord that connect to scratched areas of the skin. Furthermore, scratching behaviors and astrogliosis were partially dependent on the astrocyte transcription factor STAT3. Perhaps most intriguing, while STAT3 knockout animals displayed reduced chronic scratching behavior, their acute itch responses remained intact, suggesting that this transcription factor plays a key role in the transition to a chronic itch state. LCN2, a protein involved in intracellular transport, was also identified as a possible downstream target of STAT3 responsible for mediating its effects(Shiratori-Hayashi et al., 2015). The mechanisms by which changes in spinal and central signaling mediate the development of chronic itch remain a fascinating and active area of ongoing research.

Conclusion

A great deal of progress has been made in understanding the neuroimmune mechanisms of chronic itch in recent years. However, a number of open questions remain: What are the relative contributions of neurons and immune cells to chronic itch pathophysiology? Some of the same cell types implicated in chronic itch are also linked to chronic pain. What makes these cells behave differently in one disease context versus another? Are the neuroimmune signaling pathways in atopic dermatitis and psoriasis conserved in other inflammatory itch conditions? Will effective therapeutics require the blockade of both neuronal and immune cell pathways?

While early therapeutics, such as cyclosporine and steroids, have focused on broad immunosuppression, incomplete itch relief, remission, lack of penetrance, toxicity and side effects make these types of treatments poor options for long term use. A number of particularly exciting recent advances have been made in the area of biologics, which block the actions of key cytokines that drive chronic itch pathology. Recent studies have shown that IL-17 or IL-23 targeting antibodies are effective in reducing symptoms in psoriasis patients(Guttman-Yassky and Krueger, 2017). Although antibody targeting has thus far proven less successful for atopic disease (Sheridan, 2018), early studies show that dupilimab, which inhibits both IL-4 and IL-13 signaling by blocking IL4Ra, looks quite promising for treating medium to severe atopic dermatitis(Simpson et al., 2016). Perhaps the effectiveness of these treatments stems from their abilities to inhibit signaling in both immune and neuronal cell types. The groundbreaking work now being done in the neuroimmune interactions driving chronic itch is sure to provide crucial insights that pave the way for new therapies.

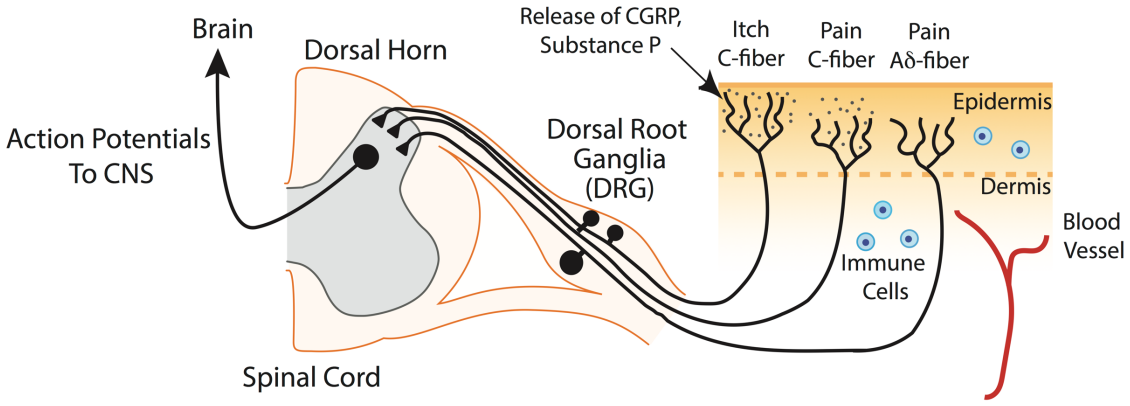

Figure 1. Somatosensory neurons have cell bodies in the dorsal root ganglia (DRG). These neurons are pseudounipolar and have two axonal branches: one branch innervates the epidermis, where environmental stimuli are transduced into action potentials, the other innervates interneurons in the dorsal horn of the spinal cord, where the signals are processed and sent to the brain. Somatosensory neurons are diverse, with itch and pain neurons innervating the epidermis and dermis and interacting with immune cells in multiple layers of the skin.

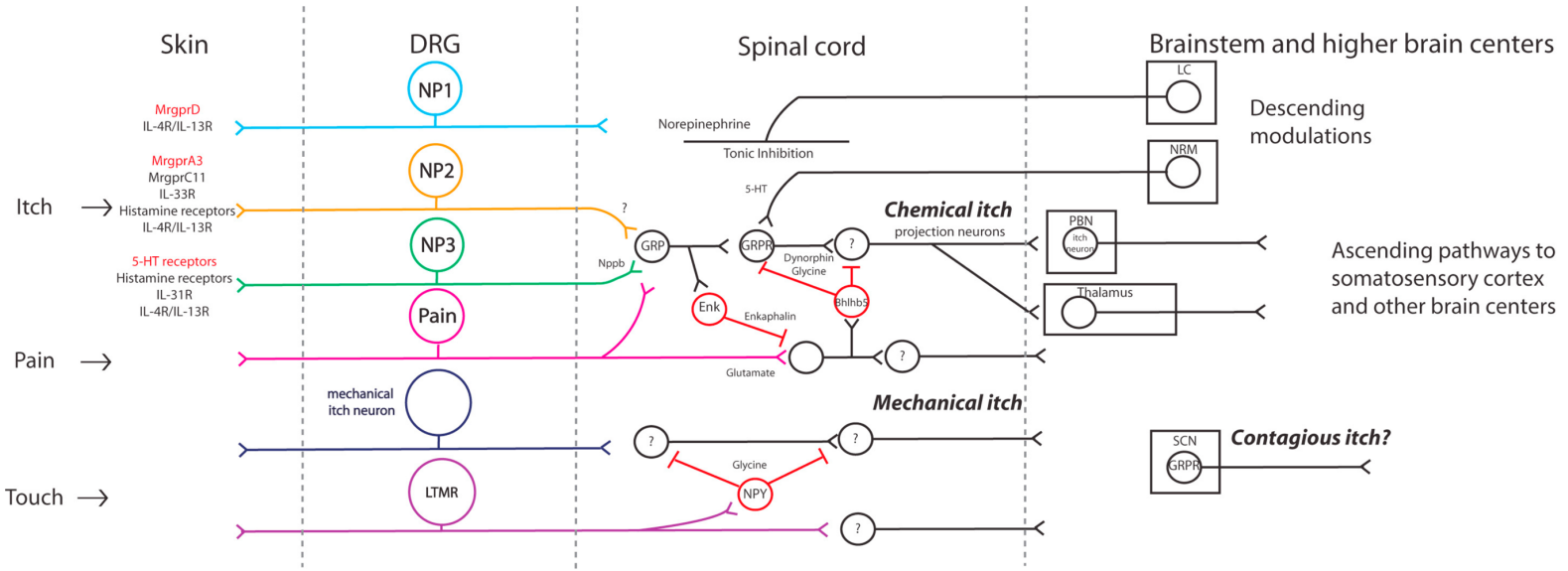

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Figure 2. Itch processing the spinal cord is highly complex. Several molecules that play key roles in itch transmission in the spinal cord have been identified, including glutamate, gastrin-releasing peptide (GRP), gastrin-releasing peptide receptor (GRPR), and dynorphin. Schematic depicts a hypothetical schematic of circuits based on the current literature. Triangular and rectangular synaptic symbols indicate excitatory and inhibitory connections. Primary somatosensory neurons in the DRG can be categorized by their gene-expression profiles. Single-cell expression profiling revealed 3 subtypes of itch neurons termed NP1, NP2, and NP3. NP1 neurons are characterized by their expression of MrgprD, NP2 by MrgprA3, and NP3 by serotonin receptors. The MrgprA3$^+$ NP2 neurons have been shown to be itch-specific neurons since activating these neurons elicit only itch behaviors. Itch sensory neurons make synaptic connections with GRP$^+$ interneurons in the spinal cord, which are in turn connected with GRPR$^+$ neurons. Ablation of GRPR signaling or GRPR$^+$ neurons completely abolished responses to itchy stimuli, indicating vital importance of this pathway in itch transmission. Information about itch stimuli in the periphery is eventually conveyed to higher brain centers by projection neurons. The parabrachial nucleus (PBN) in the brainstem serves as an important relay center in the process. Itch interacts with other sensory modalities in the spinal cord. GRP$^+$ neurons receive both itch and pain stimuli but will inhibit their own responses to high-intensity pain sensation through enkephalin signaling. Meanwhile, a painful stimulus will activate both itch and pain sensory neurons. But only pain will be perceived because itch is inhibited by pain via inhibitory interneurons in the spinal cord. One group of these interneurons is marked by the transcription factor BHLHB5. These neurons are activated by a variety of anti-itch stimuli and likely inhibit itch via the release of dynorphin and glycine. Similarly, a group of inhibitory interneurons labeled by the NPY neuropeptide were shown to mediate the inhibition of mechanical itch by light touch, though the sensory neurons and further transduction circuits of mechanical itch remain to be identified. The spinal cord dorsal horn also receives descending neuromodulation from higher brain centers. The raphe magnus (NRM) sends serotoninergic projections to the spinal cord and can stimulate GRPR$^+$ neurons and potentiate itch by co-activating HTR1A and GRPR receptors. The dorsal horn also receives tonic noradrenergic inhibition, likely from the locus coeruleus (LC). GRPR$^+$ neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus have been suggested to play a role in contagious itch.

Langerhans Cell

Nitric Oxide → **Direct neuronal activation**

Th2 Cell

IL-31, IL-4, IL-13 → **Direct neuronal activation, sensitization**

Mast Cell

proteases, serotonin, histamine, IL-13, ICAM-1 → **Direct neuronal activation, increased TRPA1 expression, increased mast cell-neuron contacts**

Eosinophil

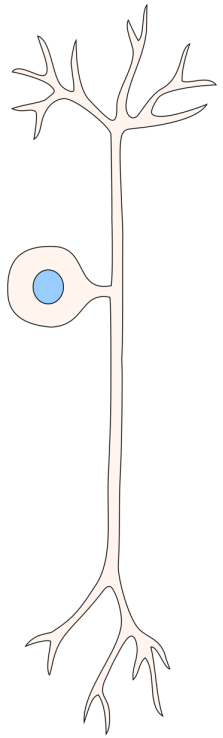
→ **Increased neurite branching**

Neutrophil

proteases, leukotrienes, reactive oxygen species, ?? → **Neuronal activation leading to scratching behaviors**

Figure 3. Immune cells modify neuronal activity to promote chronic itch. Langerhans cells, Th2-type CD4+ T cells, mast cells, and eosinophils have been shown to modulate neuronal function chronic itch disorders.

The “most wanted” neuron offenses in chronic itch



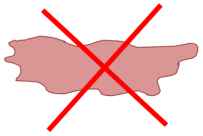
Somatosensory DRG Neuron

Denervation-evoked attenuation of psoriatic lesions →

CGRP-evoked bias towards elicitation of Th2 cell polarization →

Degranulation via MrgprB2 →

Neuron-eosinophil contacts via ICAM-1 and VCAM-1 →



Psoriasis Lesion

Langerhans Cell

Mast Cell

Eosinophil

Figure 4. Neurons act on numerous immune cells to modulate their activity in chronic itch disorders, including Langerhans cells, mast cells and eosinophils.

Molecular Mediator	Main Cellular Source	Neuronal Receptor	Ion Channel	DRG Neuron Subtypes	Cause of itch
Histamine	mast cells	H1R, H4R	TRPV1, TRPV4	NP2, NP3	insect bites, dermatitis
Serotonin (5-HT)	mast cells, keratinocytes	HTR7, HTR2	TRPA1, TRPV1, TRPV4	NP3	atopic dermatitis
Proteases	mast cells, plants	PAR2, MrgprC11	TRPA1, TRPV1	NP2	cowhage, dermatitis
TSLP	keratinocytes	TSLP receptor (IL-7Rα + TSLPR)	TRPA1		atopic dermatitis
IL-31	Th2 T helper cells	IL-31 receptor (IL-31Rα + OSMR)	TRPA1, TRPV1	NP3	atopic dermatitis, T cell lymphoma
IL-33	keratinocytes	IL-33 receptor (IL-1RAcP + ST2)	TRPA1, TRPV1	NP2	allergic contact dermatitis
IL-4 and IL-13	Th2 cells, ILC2, basophils	IL-4Rα, IL-13Rα1	TRPA1, TRPV1	NP1, NP2, NP3	atopic dermatitis, chronic idiopathic pruritis
Poly I:C, Imiquimod	pathogens, drug	TLR3, TLR7			psoriasis, xerosis (dry skin)
BAM8-22 peptide	keratinocytes	MrgprC11	TRPA1, TRPV1	NP2	xerosis (dry skin)
Chloroquine	medicine in circulation	MrgprA3	TRPA1, CNO1	NP2	drug-induced itch
β-alanine	medicine in circulation	MrgprD		NP1	drug-induced itch

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Table I. A remarkable variety of pruritogens derived from epithelial cells, immune cells, and the environment activate somatsoesensory neurons and evoke itch sensations and scratching behaviors.

Chapter 2: Neutrophils promote CXCR3-dependent itch in the development of atopic dermatitis

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INTRODUCTION

Chronic itch is a debilitating disorder that affects millions of people worldwide (Dalgard et al., 2015; Matterne et al., 2011; Mollanazar et al., 2016). It is a symptom of a number of skin diseases and systemic disorders, as well as a side effect of a growing list of medications. Like chronic pain, chronic itch can also be a disease in and of itself (Berger et al., 2013; Stumpf and Ständer, 2013). Unlike acute itch, which can facilitate removal of crawling insects, parasites, or irritants, persistent scratching in chronic itch disorders damages skin, leading to secondary infection, disfiguring lesions, and exacerbation of disease severity (Mack and Kim, 2018; Yosipovitch and Bernhard, 2013). One of the most common chronic itch disorders is atopic dermatitis (AD; commonly known as eczema), which affects fifteen million people in the US alone. Severe AD often leads to the atopic march, where chronic itch and inflammation progress to food allergy, allergic rhinitis, and asthma.

Little is known about the underlying mechanisms that drive chronic itch pathogenesis. As such, studies of human chronic itch disorders have sought to identify candidate mechanisms of disease progression. A number of studies have identified biomarkers and disease genes in human atopic dermatitis (AD) itch lesions (BS et al., 2012; Ewald et al., 2017; Guttman-Yassky et al., 2009; Jabbari et al., 2012; Li et al., 2014; Suárez-Fariñas et al., 2015). Indeed, a recent study compared the transcriptomes of healthy skin to itchy and non-itchy skin from psoriasis and AD patients, revealing dramatic changes in expression of genes associated with cytokines, immune cells, epithelial cells, and sensory neurons (Nattkemper et al., 2018). However, due to the difficulty in staging lesion development and obtaining staged samples from patients, there is currently no temporal map of when individual molecules and cell types contribute to chronic itch pathogenesis. Furthermore, the use of human patient data does not allow for rigorous mechanistic study of how disease genes contribute to chronic itch. To this end, we used a well-characterized inducible animal model of itch to define where, when, and how these genes identified from patient data contribute to chronic itch pathogenesis.

We employed the MC903 mouse model of AD and the atopic march (Dai et al., 2017; Li et al., 2009a; 2006; Moosbrugger-Martinz et al., 2017; Zhang et al., 2009) to provide a framework in which to identify the molecules and cells that initiate the development of

atopic itch. The MC903 model is ideal for this approach because of its well-defined time course of lesion development and scratching onset (Li et al., 2006; Morita et al., 2015; Oetjen et al., 2017; Zhang et al., 2009). While MC903 has been widely used to study the chronic phase of AD (Kim et al., 2013; 2014; Li et al., 2006; 2009b; Naidoo et al., 2018; Oetjen et al., 2017; Zhang et al., 2009), we hypothesized that MC903 could also be used to define the early mechanisms underlying the development of chronic itch, beginning with healthy skin. We performed RNA-seq of skin at key time points in the model and complemented this approach with measurements of itch behavior and immune cell infiltration. The primary goal of our study was to identify the inciting molecules and cell types driving development of chronic itch. To that end we found that infiltration of neutrophils into skin is required for development of chronic itch behaviors. Additionally, we demonstrate that early hyperinnervation of skin, and upregulation of itch signaling molecules and activity-induced genes in neurons are also dependent on neutrophils. Finally, we identify CXCL10/CXCR3 signaling as a key link between infiltrating neutrophils and sensory neurons that drives itch behaviors.

RESULTS

MC903 triggers rapid changes in expression of skin barrier, epithelial cell-derived cytokine, and axon guidance genes

Although a variety of AD- and chronic itch-associated genes have been identified, when and how they contribute to disease pathogenesis is unclear. Using RNA-seq of MC903-treated skin, we observed distinct temporal patterns by which these classes of genes were differentially expressed across the first eight days of the model (Figure 1A-B, Figure 1-Figure Supplement 1A). Proteases (*Klk6*, *Klk13*, among others) and skin barrier genes (*Cdhr1*) changed as early as six hours after the first treatment, before mice begin scratching (Figure 1A). Increased protease activity in AD skin is thought to promote breakdown of the epidermal barrier and release of inflammatory cytokines from keratinocytes (Steinhoff et al., 2003; Yosipovitch, 2004). One such cytokine, thymic stromal lymphopoetin (TSLP) is a key inducer of the Type 2 immune response, which is characteristic of human AD and the MC903 model, via signaling in $CD4^+$ T cells, basophils, and other immune cells (Briot et al., 2009; Demehri et al., 2009; Gao et al., 2010; Kim et al., 2013; Li et al., 2009a; Zhang et al., 2009). Beginning at day two, before any significant itch-evoked scratching (Figure 1A), immune cell infiltration (Figure 1E-G, Figure 1-Figure Supplement 2A-C), or skin lesions (data not shown) were observed, we saw increases in *Tslp*, as well as several other epithelial-derived cytokines, including the neutrophil chemoattractant genes *Cxcl1, Cxcl2, Cxcl3,* and *Cxcl5* (Figure 1D). To ask whether upregulation of these chemokine genes was dependent on protease activity, we treated human keratinocytes with the Protease Activated Receptor 2 agonist SLIGRL. SLIGRL treatment triggered increased expression of several of these chemokine genes, including *IL-8*, the human ortholog of mouse *Cxcl1/Cxcl2,* and *CXCL2* (Figure 1-Figure Supplement 3A). These increases occurred after a few hours of exposure to SLIGRL, suggesting that increased protease

activity can rapidly trigger increases in neutrophil chemokines in skin, similar to what we observe in MC903-treated mouse skin.

Unexpectedly, we observed early changes in a number of transcripts encoding neuronal outgrowth factors (*Ngf*, *Artn*) and axon pathfinding molecules (*Slit1*, *Sema3d, Sema3a*), some of which are directly implicated in chronic itch (Hidaka et al., 2016; Kou et al., 2012; Tominaga et al., 2008; Tominaga and Takamori, 2014); Figure 1-Figure Supplement 4A), prior to when mice began scratching. We thus used immunohistochemistry (IHC) of whole-mount skin to examine skin innervation at this time point. We saw increased innervation of lesions at day two but not day one of the model (Figure 1H-I, Figure 1-Figure Supplement 5A). This increase came as a surprise because such changes had previously only been reported in mature lesions from human chronic itch patients (Haas et al., 2010; Kamo et al., 2013; Nattkemper et al., 2018; Oaklander and Siegel, 2005; Pereira et al., 2016; Schüttenhelm et al., 2015; Tominaga et al., 2009). Our findings suggest that early hyperinnervation is promoted by local signaling in the skin and is independent of the itch-scratch cycle.

Neutrophils are the first immune cells to infiltrate AD skin

At day five, mice exhibited robust itch behaviors and stark changes in a number of AD disease genes (Figure 1A-B). For example, loss-of-function mutations in filaggrin (*FLG)* are a major risk factor for human eczema (Palmer et al., 2006). Interestingly, *Flg2* levels sharply decreased by this time point. In parallel, we saw a continued and significant elevation in neutrophil and basophil chemoattractant genes (*Cxcl1,2,3,5*, and *Tslp*, Figure 1D). Using flow cytometry, we determined that neutrophils were the most highly increased cell type in the skin at day five (Figure 1E). It was not until day eight that we observed the classical AD-associated immune signature (Gittler et al., 2012) in the skin, with upregulation of *Il4, Il33* and other Th2-associated genes (Figure 1B, Figure 1D). We also observed increases in the T cell chemoattractants *Cxcl9, Cxcl10,* and *Cxcl11* (Figure 1D), which are thought to be hallmarks of chronic AD lesions in humans (Mansouri and Guttman-Yassky, 2015; Oetjen and Kim, 2018). Neutrophils and a number of other immune cells that started to infiltrate on day five were significantly elevated in skin by day eight, including basophils (Figure 1F), $CD4^+$ T cells (Figure 1G), inflammatory monocytes (Figure 1-Figure Supplement 2A), and mast cells (Figure 1-Figure Supplement 2B), but not eosinophils (Figure 1-Figure Supplement 2C).

$CD4^+$ T cells are ubiquitous in human AD lesions (Guttman-Yassky and Krueger, 2017) and have been shown to play a key role in promoting chronic AD itch and inflammation (Oetjen et al., 2017). More specifically, they are implicated in IL4Rα-dependent sensitization of pruriceptors in the second week of the MC903 model (Oetjen et al., 2017). Thus, we were quite surprised to find that itch behaviors preceded significant $CD4^+$ T cell infiltration. Therefore, neutrophils drew our attention as potential early mediators of MC903 itch. While neutrophil infiltration is a hallmark of acute inflammation, it remains unclear whether neutrophils contribute to the pathogenesis of chronic itch. Moreover, neutrophils release known pruritogens, including proteases

and/or histamine, and secrete inflammatory lipids and cytokines that sensitize and/or activate pruriceptors (Dong and Dong, 2018; Hashimoto et al., 2018). Increased levels of PGE_2 and the neutrophil-specific lipid LTB_4 have also been reported in skin of AD patients (Fogh et al., 1989). Indeed, by mass spectrometry, we observed increases in several of these inflammatory lipids, PGD_2 and PGE_2, as well as LTB_4 and its precursor 5-HETE (Figure 1-Figure Supplement 6A). Thus, we next tested the requirement of neutrophils to itch in the MC903 model.

Neutrophils are required for early itch behaviors in the MC903 model of AD

We first asked whether neutrophils were required for MC903-evoked itch by depleting neutrophils, the earliest and largest population of infiltrating immune cells in this chronic itch model. Depletion of neutrophils by daily injections of an anti-Gr1 (aGr1) antibody (Ghasemlou et al., 2015; Kim et al., 2009; Sivick et al., 2014) dramatically attenuated itch-evoked scratching through the first eight days of the model (Figure 2A). Consistent with a key role for neutrophils in driving chronic itch, our depletion strategy significantly and selectively reduced circulating and skin infiltrating neutrophils on days five and eight, days on which control, but not depleted mice, scratched robustly (Figure 2B; Figure Supplement 1A-C). In contrast, basophils and $CD4^+$ T cells continued to infiltrate the skin following aGr1 treatment (Figure 2C-D), suggesting that these cells are not required for early MC903 itch.

We next used the cheek model of acute itch (Shimada and LaMotte, 2008) to ask whether neutrophil recruitment is sufficient to trigger itch. As expected, we observed significant and selective recruitment of neutrophils to cheek skin within 15 minutes after CXCL1 injection (Figure 2-Figure Supplement 2A-B). CXCL1 injection also triggered robust scratching behaviors (Figure 2E) on a similar time course to neutrophil infiltration (Figure 2 - Figure Supplement 2B). Thus, we depleted neutrophils with aGr1 to determine whether neutrophils were required for CXCL1-evoked acute itch. Indeed, aGr1-treatment resulted in a dramatic loss of CXCL1-evoked itch behaviors (Figure 2C). This effect was specific to neutrophil-induced itch as injection of chloroquine, a pruritogen that directly activates sensory neurons, triggered robust scratching in aGr1-treated animals (Figure 2-Figure Supplement 3A). Given that CXCL1 has been shown to directly excite and/or sensitize sensory neurons (Deftu 2017,2018), it is possible that the CXCL1 mechanism in itch may also involve neuronal pathways. However, our results show that CXCL1-mediated neutrophil infiltration is sufficient to drive acute itch behaviors, and that neutrophils are necessary for itch in the MC903 model.

We also examined the MC903-evoked itch behaviors of mice deficient in *Crlf2*, the gene encoding the TSLP receptor ($TSLPR^{-/-}$ mice; (Carpino et al., 2004). TSLPR is expressed by both immune cells and sensory neurons and is a key mediator of atopic dermatitis in human and mouse models. Surprisingly, MC903-treated $TSLPR^{-/-}$ mice displayed robust scratching behaviors during the first eight days of the model (Figure 2F). In contrast to our results in aGR1-injected mice, $TSLPR^{-/-}$ mice displayed robust neutrophil infiltration (Figure 2G), but a complete loss of basophil and $CD4^+$ T cell infiltration into the skin

(Figure 2H-I). These results suggest that basophils and T cells are not required for early itch and further support an inciting role for neutrophils. Previous studies have shown that TSLP drives the expression of Type 2 cytokines and related immune cells that promote itch and inflammation in mature AD skin lesions and in the second week of the MC903 AD model (Briot et al., 2009; Demehri et al., 2009; Li et al., 2009a; Zhang et al., 2009). Consistent with a later stage role for TSLP signaling in AD, we did observe a significant reduction in itch-evoked scratching in $TSLPR^{-/-}$ mice in the second week of the model (Figure 2F). Thus, our data support a model in which neutrophils are necessary for initiation of AD and itch behaviors early in the development of AD, while TSLPR signaling mediates the recruitment of basophils and $CD4^{+}$ T cells to promote later stage itch and chronic inflammation.

MC903 drives rapid and robust changes in the peripheral and central nervous systems

But how do neutrophils drive MC903 itch? Itchy stimuli are detected and transduced by specialized subsets of peripheral somatosensory neurons. Thus, to answer this question we first profiled the transcriptional changes in somatosensory neurons in the MC903 model, which were previously unstudied. In general, little is known regarding neuronal changes in chronic itch. Our initial examination of early hyperinnervation and changes in axon guidance molecules in skin suggested that neurons are indeed affected early on in the MC903 model, before the onset of itch-evoked scratching behaviors. In contrast to the skin, where we saw many early transcriptional changes, we did not see any significant transcriptional changes in the trigeminal ganglia (TG) until five days after the first treatment, and in total only 84 genes were differentially expressed through the eighth day (Figure 3A-B). These hits included genes related to excitability of itch sensory neurons (Dong and Dong, 2018; Usoskin et al., 2015), neuroinflammatory genes (Takeda et al., 2009) and activity-induced or immediate early genes (Figure 3A). Interestingly, we observed enrichment of neuronal markers expressed by one specific subset of somatosensory neurons that are dedicated to itch (*Il31ra, Osmr, Trpa1, Cysltr2,* and *Nppb* (Solinski et al., 2019; Usoskin et al., 2015), termed “NP3” neurons. Similar to what has been reported in mouse models of chronic pain, we observed changes in neuroinflammatory (*Bdnf, Nptx1, Nptx2, Nptxr*) and immune genes (*Itk, Cd19, Rag, Tmem173*). However, these transcriptional changes occurred just a few days after itch onset, in contrast to the slow changes in nerve injury and pain models that occur over weeks (Scholz and Woolf, 2007), indicating that neuropathic changes may occur sooner than previously thought. These changes occurred in tandem with the onset of scratching behaviors (Figure 1C), suggesting that the early molecular and cellular changes we observed by this time point may be important for development of itch-evoked scratching.

The changes we observed in immune-related genes in the TG were suggestive of infiltration or expansion of immune cell populations, which has been reported in models of nerve injury and chronic pain, but has never been reported in chronic itch. To validate our observations, we used IHC to ask whether $CD45^{+}$ immune cells increase in the TG.

We observed a significant increase in immune cell counts at day eight but not day five (Figure 3C-F, Figure 3-Figure Supplement 1A-D). Because we observed such dramatic expression changes in the TG on day eight of the model, we postulated that the CNS may also be affected at this time point. Thus, we performed RNA-seq on spinal cord segments that innervate the rostral back skin of MC903-treated mice. To date, only one study has examined changes in the spinal cord during chronic itch. They showed that upregulation of the STAT3-dependent gene *Lcn2* occurred three weeks after induction of chronic itch and was essential for sustained scratching behaviors (Shiratori-Hayashi et al., 2015). Surprisingly, we saw upregulation of *Lcn2* on day eight of the MC903 model and, additionally, we observed robust upregulation of immediate early genes (*Fos, Junb,* Figure 3G), suggesting that MC903 itch drives activity-dependent changes in the spinal cord as early as one week after beginning treatment. Together our findings show that sustained itch and inflammation can drive changes in the PNS and CNS much sooner than previously thought, within days rather than weeks after the onset of scratching. We next set out to ask how loss of neutrophils impacted the molecular changes observed in skin and sensory neurons in the MC903 model, and which of these changes might contribute to neutrophil-dependent itch.

Neutrophils are required for upregulation of select itch- and atopic-related genes, including the itch-inducing chemokine CXCL10

To ask how neutrophils might promote itch in the MC903 model, we examined the transcriptional changes in skin and sensory ganglia isolated from non-itchy neutrophil-depleted animals and from the TSLPR$^{-/-}$ mice, which scratched robustly. In both the neutrophil-depleted and TSLPR$^{-/-}$ skin, several AD-associated cytokines were similarly dysregulated, including *Il33* (Figure 4A, Figure 4-Figure Supplement 1A). Expression of epithelial-derived cytokines and chemokines *Tslp*, *Cxcl1*, *Cxcl2*, *Cxcl3*, and *Cxcl5* were unaffected by either loss of TSLPR or neutrophil depletion (Figure 4B), suggesting these molecules are produced by skin cells even when the MC903-evoked immune response is compromised. Among the hundreds of MC903-dependent genes we examined, only a handful of genes were uniquely affected by aGr1 treatment. One such gene was *Cxcl10*, a chemokine known to be released by skin epithelial cells, neutrophils, and other myeloid cells (Hashimoto et al., 2018; Ioannidis et al., 2016; Kanda et al., 2007; Koga et al., 2008; Michalec et al., 2002; Padovan et al., 2002; Tamassia et al., 2007). *Cxcl10* expression was increased in TSLPR$^{-/-}$ but not neutrophil-depleted skin (Figure 4B, Figure 4-Figure Supplement 1A). CXCL10 has been previously shown to drive acute itch in a model of allergic contact dermatitis via CXCR3 signaling in sensory neurons (Qu et al., 2015), and is elevated in skin of AD patients (Mansouri and Guttman-Yassky, 2015). Expression of *Cxcl9* and *Cxcl11*, two other CXCR3 ligands that are elevated in AD but have an unknown role in itch, was also decreased in AD skin of neutrophil-depleted mice (Figure 4B).

CXCR3 signaling is necessary for MC903-evoked chronic itch

We hypothesized that neutrophil-dependent upregulation of CXCL10 activates sensory neurons to drive itch behaviors. Consistent with this model, neutrophil depletion

attenuated the expression of activity-induced immediate early genes (*Vgf, Junb*) in the TG, suggestive of neutrophil-dependent sensory neuronal activity in the MC903 model (Figure 4C, Figure 4-Figure Supplement 1B). We found that neutrophils also contributed to other sensory neuronal phenotypes in the model. For example, we observed that expression of *Lcn2*, a marker of neuropathic itch, and activity-induced genes *Fos* and *Junb* were not increased in spinal cord isolated from neutrophil-depleted animals after MC903 treatment, indicating that neutrophil-dependent scratching behaviors may indeed drive changes in the CNS (Figure 4D). We also observed that neutrophil-depleted animals displayed no skin hyperinnervation at day two (Figure 4E). This result was surprising because we did not observe significant neutrophil infiltration at this early time point, but these data suggest that low numbers of neutrophils are sufficient to mediate these early effects.

To test our model wherein CXCL10 activation of CXCR3 drives neutrophil-dependent itch, we first asked whether this CXCR3 ligand is in fact released in MC903-treated skin. We performed ELISA on cheek skin homogenate and found that CXCL10 protein was increased in MC903-treated skin, but not in skin from neutrophil-depleted mice (Figure 4F). To test whether CXCR3 signaling directly contributes to MC903-evoked itch, we asked whether acute blockade of CXCR3 signaling using the antagonist AMG 487 (Qu et al., 2015) affected scratching behaviors in the MC903 model. We found that the CXCR3 antagonist strongly attenuated scratching behaviors on days five, eight, and twelve (Figure 4G), with the greatest effect at day eight. In contrast, CXCR3 blockade did not attenuate scratching behaviors in naive mice injected with the pruritogen chloroquine (Figure 4G), demonstrating that CXCR3 signaling contributes to chronic itch but is not required for scratching in response to acute pruritogens. Thus, we propose that neutrophils promote chronic itch via upregulation of CXCL10 and subsequent activation of CXCR3 (Figure 5).

DISCUSSION

There is great interest in unraveling the neuroimmune interactions that promote acute and chronic itch. Here, we show that neutrophils are essential for the early development of MC903-evoked itch. We further show that the acute recruitment of neutrophils to the skin is sufficient to drive itch behaviors within minutes of infiltration. While neutrophils are known to release a variety of pruritogens, their roles in itch and AD were not studied (Hashimoto et al., 2018). Only a few studies have even reported the presence of neutrophils in human AD lesions (BS et al., 2012; Koro et al., 1999; Mihm et al., 1976; Shalit et al., 1987). Neutrophils have been implicated in psoriatic inflammation and inflammatory pain (Carreira et al., 2013; Cunha et al., 2008; Pinho-Ribeiro et al., 2018; Sumida et al., 2014), where they are thought to rapidly respond to tissue injury and inflammation (Kim and Luster, 2015; Kolaczkowska and Kubes, 2013), but they have not been directly linked to itch. A previous study showed that the neutrophil chemoattractant *Cxcl1* was elevated in a mouse model of dry skin itch (Wilson et al.,

2013a). While tissue damage can trigger upregulation of neutrophil chemoattractants and neutrophil infiltration, *Cxcl1* expression was upregulated both in scratched and unscratched itchy skin, suggesting that upregulation of CXCL1, and subsequent neutrophil infiltration, is not simply a consequence of tissue damage or the itch-scratch cycle.

TSLP signaling triggers development of a Type 2 immune response, which is crucial for the progression of atopic disease. For example, Th2 cytokines, most notably IL-4 and IL-13, are thought to drive the transition from the acute to chronic phase of human AD, and IL4Rα signaling was recently shown to mediate inflammation and itch in the second week of the MC903 AD mouse model (Oetjen et al., 2017). Interestingly, the same study showed that IL-4 sensitizes the population of itch sensory neurons that responds to TSLP. Based upon their observation, and because TSLP is induced early on in the MC903 model, we expected TSLP to contribute to early MC903 itch. We were surprised to find that TSLPR signaling is dispensable for itch behaviors in the first week of the model, and is only required for itch in the second week. In light of our observation that neutrophils are required for early MC903 itch, and that TSLPR signaling is necessary for increased IL-4 production, we hypothesize that TSLPR signaling plays a key role in later, IL-4-dependent itch, whereas early itch is independent of IL-4. Our findings align well with a recent study showing that TSLP and IL4 act on overlapping sensory neuron populations and that neuronal IL4Rα promotes itch during the second week of the MC903 model (Oetjen et al., 2017).

Given the large magnitude of the itch deficit in the neutrophil-depleted mice, we were surprised to find fewer expression differences in MC903-dependent, AD-associated genes between neutrophil depleted and non-depleted mice than were observed between WT and TSLPR$^{-/-}$ mice. One of the few exceptions were the Th1-associated genes *Cxcl9/10/11* (Brunner et al., 2017; Ewald et al., 2017). We found that induction of these genes and CXCL10 protein was completely dependent on neutrophils. While our results do not identify the particular cell type(s) responsible for neutrophil-dependent CXCL10 production, a number of cell types present in skin have been shown to produce CXCL10, including epithelial keratinocytes, myeloid cells, and sensory neurons (Flier et al., 2001; Hashimoto et al., 2018; Ioannidis et al., 2016; Kanda et al., 2007; Koga et al., 2008; Michalec et al., 2002; Padovan et al., 2002; Steain et al., 2011; Tamassia et al., 2007). We also observed striking differences in neutrophil-dependent gene expression in the spinal cord, where expression of activity-induced genes and the chronic itch gene *Lcn2* were markedly attenuated by loss of neutrophils. Our observations newly implicate neutrophils in setting the stage for the acute-to-chronic itch transition by triggering molecular changes necessary to develop a chronic, itchy lesion.

Additionally, we demonstrate a novel role of CXCR3 signaling in MC903-induced itch. The CXCR3 ligand CXCL10 contributes to mouse models of acute and allergic itch (Liu et al., 2016; Qu et al., 2015); however, its role in chronic itch was previously unknown. Our results show that CXCL10 upregulation is dependent on neutrophils and that

CXCR3 blockade dramatically attenuates itch behaviors. Our findings are in alignment with a recent study showing that dupilumab, a new AD drug that blocks IL4Rα, a major downstream effector of the TSLP signaling pathway, does not significantly reduce CXCL10 levels in human AD lesions (Hamilton et al., 2014). Taken together, this suggests that the TSLP-IL-4 and neutrophil-CXCL10 pathways are not highly interdependent, and supports our findings that *Il4* is robustly upregulated in the absence of neutrophils. Additionally, targeting IL4Rα has been successful in treating itch and inflammation in some, but not all, AD patients (Simpson et al., 2016). We propose that biologics or compounds targeting neutrophils and/or the CXCR3 pathway may be useful for AD that is incompletely cleared by dupilumab monotherapy. Drugs targeting neutrophils are currently in clinical trials for the treatment of psoriasis, asthma, and other inflammatory disorders. For example, MDX-1100, a biologic that targets CXCL10, has already shown efficacy for treatment of rheumatoid arthritis in phase II clinical trials (Yellin et al., 2012). While rheumatoid arthritis and AD have distinct etiologies (Scott et al., 2010), our work indicates that CXCL10 or CXCR3 may be promising targets for treating chronic itch. Our findings may be applicable to other itch disorders such as psoriasis and ACD where neutrophil chemoattractants and/or CXCL10 are also elevated. Overall, our data suggest that neutrophils incite early itch and inflammation in the development of AD through several mechanisms, including: 1) directly triggering itch upon infiltration into the skin (e.g. by release of known sensitizing/itchy lipids such as PGE_2 and LTB_4, Figure 4) and, 2) indirectly triggering itch by altering expression of known pruritogens (e.g. induction of *Cxcl10* expression; (Flier et al., 2001; Hashimoto et al., 2018; Ioannidis et al., 2016; Kanda et al., 2007; Koga et al., 2008; Michalec et al., 2002; Padovan et al., 2002; Tamassia et al., 2007). These direct and indirect mechanisms for neutrophil-dependent itch may explain why neutrophils have a dramatic effect on scratching behaviors on not only day eight but also day five of the model, when neutrophils are recruited in large numbers, but CXCR3 ligands are not as robustly induced.

More generally, our study provides a framework for understanding how and when known human disease genes contribute to the distinct stages of chronic itch pathogenesis. Our analysis of MC903-evoked transcriptional changes suggests we may be able to extend findings in the model not only to atopic dermatitis, but also to related disorders, including specific genetic forms of atopy. For example, we provide evidence that the MC903 model may also model the filaggrin loss-of-function mutations, which are a key inciting factor in human heritable atopic disease (Palmer et al., 2006; Schuttelaar et al., 2009). There are many rich datasets looking at mature patient lesions (BS et al., 2012; Ewald et al., 2017; Guttman-Yassky et al., 2009; Jabbari et al., 2012; Li et al., 2014; Suárez-Fariñas et al., 2015) and datasets for mature lesions in other mouse models of chronic itch (Liu et al., 2016; Oetjen et al., 2017; Swindell et al., 2011; 2017). Our study adds a temporal frame of reference to these existing datasets to probe the function of human disease genes in AD in greater detail. Furthermore, we have mapped the time course of gene expression changes in primary sensory ganglia and spinal cord during chronic itch development. We show that the MC903 model

recapitulates several hallmarks of neuropathic disease on a time course much shorter than has been reported for chronic itch, or chronic pain. Nervous system tissues are extremely difficult to obtain from human AD patients, and thus little is known regarding the neuronal changes in chronic itch disorders in both mouse models and human patients. Our findings can now be compared to existing and future datasets examining neuronal changes in chronic pain, diabetic neuropathy, shingles, neuropathic itch, psoriasis, and other inflammatory disorders where neuronal changes are poorly understood but clearly contribute to disease progression. The early changes we see in skin innervation, sensory ganglia, and spinal cord dovetail nicely with recent studies examining neuroimmune interactions in other inflammatory conditions (Baral et al., 2018; Blake et al., 2018; Pinho-Ribeiro et al., 2018), which all implicate early involvement of sensory neurons in the pathogenesis of inflammatory diseases.

MATERIALS AND METHODS

Mouse studies

All mice were housed in standard conditions in accordance with standards approved by the Animal Care and Use Committee of the University of California Berkeley (12 hr light-dark cycle, 21 ˚C). Wild-type C57Bl6 mice were obtained from Charles River or Jackson Laboratories or raised in-house. TSLPR$^{-/-}$ mice were kindly provided by Dr. Steven Ziegler (*Crlf2*^{tm1Jni}; (Carpino et al., 2004) and were backcrossed onto C57Bl6. All experiments were performed under the policies and recommendations of the International Association for the Study of Pain and approved by the University of California Berkeley Animal Care and Use Committee. Genotypes were assessed using standard PCR.

MC903 model of atopic dermatitis

MC903 (R&D Systems) was applied to the mouse cheek (20 µl of 0.2 mM in ethanol) once per day for 1-12 days. 100% ethanol was used as a vehicle control. 3-7 days prior to the beginning of treatment, mice were singly housed and their cheeks were shaved. All MC903 studies were performed on 8-12 week old age-matched mice. Behavior, RNA-seq, flow cytometry, and immunohistochemistry were performed on days 1, 2, 3, 5, 8 and/or 12. Behavioral scoring was performed while blind to experimental condition and mouse genotype. Itch behavior was scored for the first 30 min. For AMG 487 experiments in the MC903 model, 50 μL 3.31 mM AMG 487 (Tocris) or 20% HPCD-PBS vehicle was injected subcutaneously one hour prior to recording behavior (Qu et al., 2015). Both bout number and length were recorded.

MC903 RNA isolation and sequencing

At Days 1 (six hours post-treatment), 2, 5, and 8 post-treatment, mice treated with MC903 or vehicle were euthanized via isoflurane and cervical dislocation. Cheek skin was removed, flash-frozen in liquid nitrogen, and cryo-homogenized with a mortar and pestle. Ipsilateral trigeminal ganglia were dissected and both skin and trigeminal ganglia

were homogenized for three minutes (skin) or one minute (TG and spinal cord) in 1 mL RNAzol RT (Sigma-Aldrich). Thoracic spinal cord was dissected from mice treated with 40 μL MC903 or ethanol on the shaved rostral back and homogenized in 1 mL RNAzol. Large RNA was extracted using RNAzol RT per manufacturer's instructions. RNA pellets were DNase treated (Ambion), resuspended in 50 μL DEPC-treated water, and subjected to poly(A) selection and RNA-seq library preparation (Apollo 324). Single-end read sequencing (length = 50 bp) was performed by the QB3 Vincent G. Coates Genomic Sequencing Laboratory (UC Berkeley) on an Illumina HiSeq4000. See **Supplementary Table 1** for number of mice per experimental condition and number of mapped reads per sample.

MC903 RNA sequencing analysis

Reads were mapped to the mm10 mouse genome using Bowtie2 and Tophat (Langmead and Salzberg, 2012; Langmead et al., 2009). For a given time point, replicate measurements for each gene from treated and control mice were used as input for DESeq (R) and genes with $p_{\text{adjusted}} < 0.05$ (for skin and spinal cord) or $p_{\text{adjusted}} < 0.1$ (for trigeminal ganglia) for at least one time point were retained for analysis (Anders and Huber, 2010). For the skin dataset, we collated a set of AD-related immune cell markers, cytokines, atopic dermatitis disease genes, neurite outgrown/axonal guidance genes, and locally expressed neuronal transcripts, and from this list visualized genes that were significantly differentially expressed for at least one time point. For the trigeminal ganglia dataset, we plotted all genes that were significantly differentially expressed for at least one time point. Genes from these lists were plotted with clustering using heatmap2 (R).

Custom gene groups

Genes were clustered into functional groups and significance was evaluated using a permutation test. Briefly, we first tabulated the absolute value of the $\log_2$ fold change of gene expression (between MC903 and EtOH) of each gene in a given group of n genes in turn, and then we calculated the median of these fold change values, z_{true}. We then drew n random genes from the set of all genes detected in the samples and computed the median $\log_2$ fold change as above using this null set, z_{null}. Repeating the latter 10,000 times established a null distribution of median $\log_2$ fold change values; we took the proportion of resampled gene groups that exhibited ($z_{true} \geq z_{null}$) as an empirical p-value reporting the significance of changes in gene expression for a given group of n genes.

Flow Cytometry

Skin samples were collected from the cheek of mice at the indicated time points with a 4- or 6-mm biopsy punch and minced into smaller pieces with scissors. Myeloid cells were digested for 1h at 37 ˚C using Liberase (Roche)**.** At the end of the digestion, cells were washed in FACS buffer (PBS with 0.5% FCS and 2 mM EDTA) and filtered through a 70 or 100 μm cell strainer (Falcon, #352350). Cells were stained with LIVE/DEAD fixable stain Aqua (Invitrogen), then blocked with anti-CD16/32 (UCSF

Core) and stained with fluorophore-conjugated cKit-Biotin (ACK2) (secondary stain with SA-FITC), anti-CD11b-PB (M1/70, eBiosciences), Ly6C-PerCP (HK1.4), CD49b-PECy7 (DX5), CD45.2-APCCy7 (104), FceRI-PE (MAR-1), Ly6G-AF700 (1A8, all from eBiosciences). 10ml of counting beads (Invitrogen) were added after the last wash to measure absolute cell counts. $CD4^+$ T cells were digested for 30 minutes at 37˚C using Collagenase VIII (Sigma). At the end of the digestion, cells were washed in RPMI buffer (RPMI, 5% FCS, 1% penicillin-streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate). Cells were blocked with antiCD16/32 (UCSF Core) and stained with anti-CD45-APC780 (30-F11; eBiosciences), Cd11b-PECy7 (M1/70; BD Biosciences), B220-PECy7 (RA3-6B2; Tonbo Biosciences), Cd11c-PECy7 (N418; eBiosciences), CD3-FITC (145-2C11; eBiosciences), CD8-BV785 (53-6.7; Biolegend), CD4-PE (GK1.5; BD Biosciences), 647-gdTCR (GL3; Biolegend). 10μl of counting beads (Invitrogen) were added after the last wash to measure absolute cell counts, and samples were resuspended in DAPI LIVE/DEAD (Invitrogen). Single cell suspensions were analyzed on an LSR II or LSR Fortessa (BD Biosciences), and data were analyzed using FlowJo (TreeStar, v.9.9.3) software. One skin sample was excluded from analysis due to clear blood contamination. Blood samples were collected from saphenous vein or from terminal bleed following decapitation. Red blood cells were lysed using ACK lysis buffer (Gibco, #A10492-01), washed with FACS buffer (PBS with 5% FBS and 2 mM EDTA), and blocked with antiCD16/32 (UCSF Core). Cells were stained with Ly6G-PE (1A8; BD Biosciences), Cd11b-PB (M1/70, eBiosciences), Ly6C-PerCP (HK1.4, Biolegend), and aGr1-APCCy7 (RB6-8C5, eBiosciences).

Human keratinocyte RNA sequencing

Normal human epidermal keratinocytes from juvenile skin (PromoCell #C-12001) were cultured in PromoCell Keratinocyte Growth Medium 2 and passaged fewer than 5 times. Cells were treated for three hours at room temperature with 100 μM SLIGRL or vehicle (Ringer's + 0.1% DMSO). Total RNA was extracted by column purification (Qiagen RNeasy Mini Kit). RNA was sent to the Vincent J. Coates Sequencing Laboratory at UC Berkeley for standard library preparation and sequenced on an Illumina HiSeq2500 or 4000. Sequences were trimmed (Trimmomatic), mapped (hg19, TopHat) and assigned to transcripts using htseq-count. Differential gene expression was assessed using R (edgeR).

IHC of whole-mount skin

Staining was performed according to Hill et al. (Hill et al., 2018). Briefly, 8-week-old mice were euthanized and the cheek skin was shaved and tape-stripped. The removed skin was fixed overnight in 4% PFA, then washed in PBS (3X for 10 min each). Dermal fat was scraped away with a scalpel and skin was washed in PBST (0.3% Triton X-100; 3X for two hours each) then incubated in 1:500 primary antibody (Rabbit anti beta-Tubulin III: Abcam #ab18207) in blocking buffer (PBST with 5% goat serum and 20% DMSO) for 6 days at 4°C. Skin was washed as before and incubated in 1:500 secondary antibody (Goat anti-Rabbit Alexa 594; Invitrogen #R37117) in blocking buffer for 3 days at 4°C. Skin was washed in PBST, serially dried in methanol: PBS solutions,

incubated overnight in 100% methanol, and finally cleared with a 1:2 solution of benzyl alcohol: benzyl benzoate (BABB; Sigma) before mounting between No. 1.5 coverglass. Sectioned and whole mount skin samples were imaged on a Zeiss LSM 880 confocal microscope with OPO using a 20x water objective. Image analysis was performed using a custom macro in FIJI. Briefly, maximum intensity z-projections of the beta-tubulin III channel were converted to binary files that underwent edge-detection analysis. Regions were defined by circling all stained regions. Region sizes and locations were saved.

IHC of sectioned trigeminal ganglia

TG were dissected from 8 to 12 week old adult mice and post-fixed in 4% PFA for one hour. TG were cryo-protected overnight at 4°C in 30% sucrose-PBS, embedded in OCT, and then cryosectioned at 14 µm onto slides for staining. Slides were washed 3x in PBST (0.3% Triton X-100), blocked in 2.5% horse serum + 2.5% BSA PBST, washed 3X in PBST, blocked in endogenous IgG block (1:10 F(ab) anti-mouse IgG (Abcam ab6668) + 1:1000 Rat anti-mouse CD16/CD32 (UCSF MAB Core) in 0.3% PBST), washed 3X in PBST and incubated overnight at 4°C in 1:1000 primary antibody in PBST + 0.5% horse serum + 0.5% BSA. Slides were washed 3x in PBS, incubated 2 hr at RT in 1:1000 secondary antibody, washed 3X in PBS, and then incubated 30 min in 1:2000 DAPI-PBS. Slides were washed 3x in PBS and mounted in Fluoromount-G with No. 1.5 coverglass. Primary antibodies used: Mouse anti-CD45 (eBioscience #14-054-82) and Chicken anti-Peripherin (Abcam #39374). Secondary antibodies used: Goat anti-Chicken Alexa 594 (ThermoFisher #A11042) and Goat anti-Mouse Alexa 488 (Abcam #150117). DAPI (ThermoFisher #D1306) was also used. Imaging of TG IHC experiments was performed on an Olympus IX71 microscope with a Lambda LS-xl light source (Sutter Instruments). For TG IHC analysis, images were analyzed using automated scripts in FIJI (ImageJ) software. Briefly, images were separated into the DAPI, CD45, and Peripherin channels. The minimum/maximum intensity thresholds were batch-adjusted to pre-determined levels, and adjusted images were converted to binary files. Regions were defined by circling all stained regions with pre-determined size-criteria. Region sizes and locations were saved. All ImageJ macros are available upon request. All scripts are available upon request.

Neutrophil depletion

Neutrophils were acutely depleted using intraperitoneal injection with 250 *µ*g aGR1 in PBS (clone RB6-8C5, a gift from D. Portnoy, UC Berkeley, or Biolegend), 16-24 hours before behavioral and flow cytometry experiments. Depletion was verified using flow cytometry on blood collected post-mortem. For longer depletion experiments using the MC903 model, mice were injected (with 250 *µ*g aGR1 in PBS or PBS vehicle, i.p.) beginning one day prior to MC903 administration and each afternoon thereafter through day 7 of the model, and blood was collected via saphenous venipuncture at days 3, 5, or by decapitation at day 8 to verify depletion.

CXCL10 ELISA measurements in skin

Neutrophil-depleted or uninjected mice were treated with MC903 or ethanol for 7 days. On day 8, 6mm biopsy punches of cheek skin were harvested, flash-frozen in liquid nitrogen, cryo-homogenized by mortar and pestle, and homogenized on ice for three minutes at maximum speed in 0.5 mL of the following tissue homogenization buffer (all reagents from Sigma unless stated otherwise): 100 mM Tris, pH 7.4; 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and 0.5% Sodium deoxycholate in ddH2O; on the day of the experiment, 200 mM fresh PMSF in 100% ethanol was added to 1mM, with 1 tablet cOmplete protease inhibitor (Roche) per 50 mL, and 5 tablets PhosSTOP inhibitor (Roche) per 50 mL buffer. Tissues were agitated in buffer for two hours at 4°C, and centrifuged at 13,000 rpm for 20 minutes at 4°C. Supernatants were aliquoted and stored at -80C for less than one week after isolation. After thawing, samples were centrifuged at 10,000 rpm for five minutes at 4°C. Protein content of skin homogenates was quantified by BCA (Thermo Scientific) and homogenates were diluted to 2 mg/mL protein in PBS and were subsequently diluted 1:2 in Reagent Diluent (R&D Systems). CXCL10 protein was quantified using the Mouse CXCL10 Duoset ELISA kit (R&D Systems; #DY466-05) according to manufacturer's instructions. Plate was read at 450 nm and CXCL10 was quantified using a seven-point standard curve (plus blank and buffer controls) and fitted with a 4-parameter logistic curve.

Acute itch behavior

Itch and acute pain behavioral measurements were performed as previously described (Morita et al., 2015; Tsunozaki et al., 2013; Wilson et al., 2013b). Mice were shaved one week prior to itch behavior. Compounds injected: 1 μg carrier-free CXCL1 (R&D systems) in PBS, 3.31 mM AMG 487 (Tocris, prepared from 100 mM DMSO stock) in 20% HPCD-PBS, 50 mM Chloroquine diphosphate (Sigma) in PBS (Chloroquine, CXCL1), along with corresponding vehicle controls. Acute pruritogens were injected using the cheek model (20 μL, i.d.) of itch, as previously described (Shimada and LaMotte, 2008). AMG 487 (50 μL) or vehicle was injected s.c. into the rostral back skin one hour prior to recording of behavior (Qu et al 2015). Behavioral scoring was performed as described above.

Lipidomics

Skin was collected from the cheek of mice post-mortem with a 6-mm biopsy punch and immediately flash-frozen in liquid nitrogen. Lipid mediators and metabolites were quantified via liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described before (Moltke et al., 2012; Sapieha et al., 2011). In brief, skin was homogenized in cold methanol to stabilize lipid mediators. Deuterated internal standards (PGE_2-d4, LTB_4-d4, 15-HETE-d8, LXA_4-d5, DHA-d5, AA-d8) were added to samples to calculate extraction recovery. LC-MS/MS system consisted of an Agilent 1200 Series HPLC, Luna C18 column (Phenomenex, Torrance, CA, USA), and AB Sciex QTRAP 4500 mass spectrometer. Analysis was carried out in negative ion mode, and lipid 30 mediators quantified using scheduled multiple reaction monitoring (MRM) mode using four to six specific transition ions per analyte.

Statistical analyses

Different control experimental conditions (*e.g.* uninjected versus PBS-injected animals) were pooled when the appropriate statistical test showed they were not significantly different (**Supplementary Table 2**). For all experiments except RNA-seq (see above), the following statistical tests were used, where appropriate: Student's t-test, one-way ANOVA with Tukey-Kramer post hoc comparison, and two-way ANOVA with Tukey Kramer or Sidak's post-hoc comparison. Bar graphs show mean ± SEM. Statistical analyses were performed using PRISM 7 software (GraphPad). For all p values, *=0.01<p<0.05, **=0.001<p<0.01, ***=0.0001<p<0.001, and ****=p<0.0001.

FIGURES

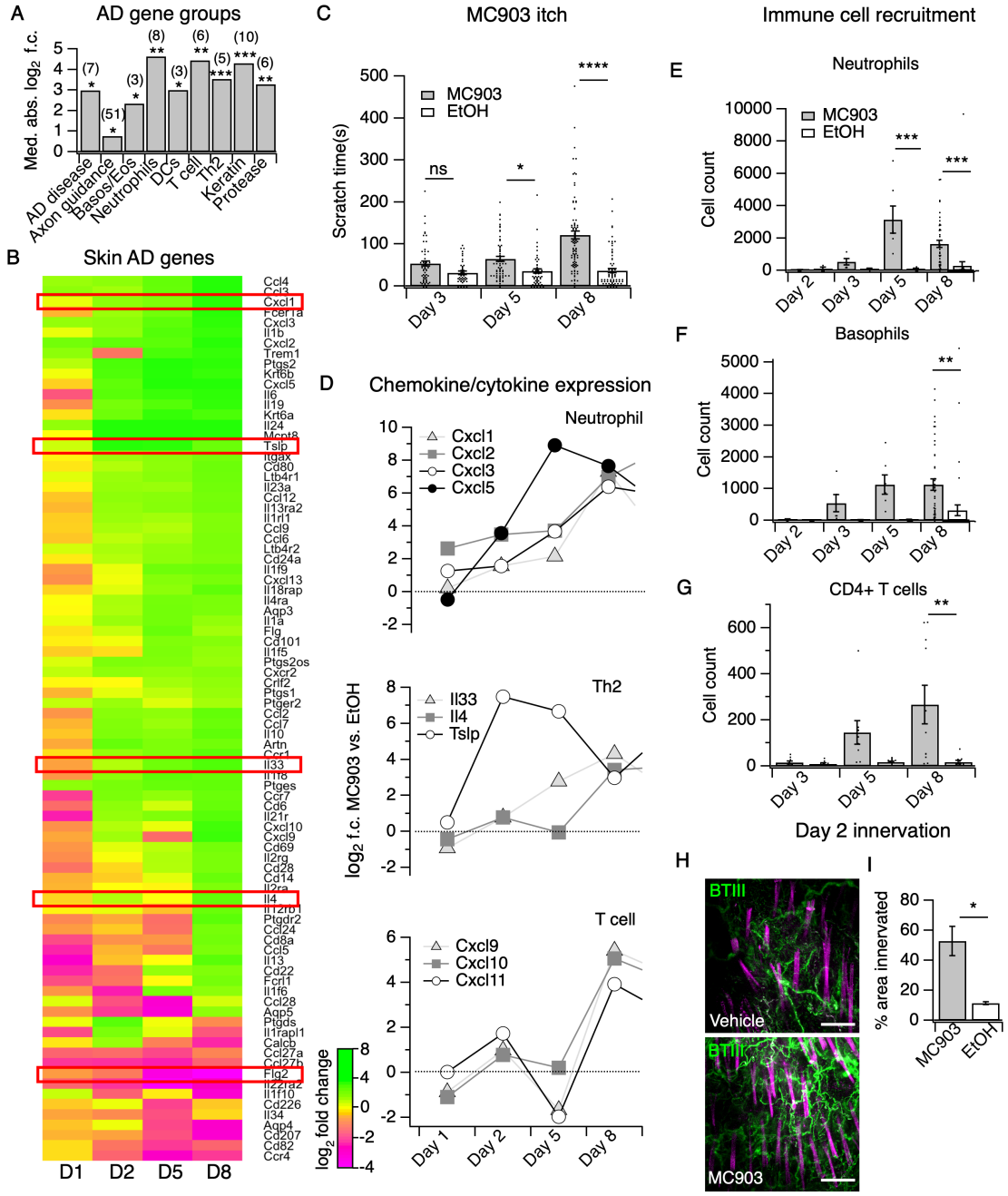


Figure 1. The MC903 model parallels the progression of human atopic disease and suggests a temporal sequence of AD pathogenesis. **A.** Exact permutation test (10,000 iterations, see Methods) for significance of mean absolute log_2 fold change in gene expression at Day 8 (MC903 vs. ethanol) of custom-defined groups of genes for indicated categories). **B.** Log_2 fold change in gene expression (MC903 vs. ethanol) in mouse skin at indicated time points for key immune and mouse/human atopic dermatitis genes that were significantly differentially expressed for at least one time point in the

MC903 model. Only genes from our initial list (see Methods) differentially expressed at corrected $p < 0.05$ and changing > 2-fold between treatments for at least one condition are shown. Green bars = increased expression in MC903 relative to ethanol; magenta = decreased expression. D1 = 6 hours post-treatment; D2 = Day 2; D5 = Day 5; D8 = Day 8. **C.** Scratching behavior of mice treated with MC903 or ethanol for indicated length of time (two-way ANOVA: ****$p_{interaction}$< 0.0001, F(2,409) = 13.25; Sidak's multiple comparisons: $p_{day\ 3}$ = 0.1309, n=62,51 mice; *$p_{day\ 5}$ = 0.0171, n=69,56 mice; ****$p_{day\ 8}$< 0.0001, n=92,85 mice). **D.** Log_2 fold change in gene expression of neutrophil chemoattractants (upper), Th2 cytokines (middle) and T cell chemoattractants (lower, from RNA-seq data). **E.** Neutrophil counts in MC903- and ethanol-treated skin at indicated time points (two-way ANOVA: **$p_{treatment}$= 0.0023, F(1,102) = 9.82; Sidak's multiple comparisons: $p_{day\ 2}$ > 0.999, n=4,4 mice; $p_{day\ 3}$ = 0.9801, n=5,5 mice; ***$p_{day\ 5}$ = 0.0003, n=6,8 mice; ***$p_{day\ 8}$ = 0.0001, n=40,38 mice). **F.** Basophil counts in MC903- and ethanol-treated skin at indicated time points (two-way ANOVA: *$p_{treatment}$= 0.0215, F(1,102) = 5.45; Sidak's multiple comparisons: $p_{day\ 2}$ > 0.999, n=4,4 mice; $p_{day\ 3}$ = 0.8566, n=5,5 mice; $p_{day\ 5}$ = 0.1280, n=6,8 mice; **$p_{day\ 8}$ = 0.0010, n=40,38 mice). **G.** $CD4^+$ T cell counts in MC903- and ethanol-treated skin at indicated time points (two-way ANOVA: **p_{time}= 0.0042, F(1,44) = 9.10; $p_{day\ 3}$ = 0.9998, n=8,6 mice; $p_{day\ 5}$ = 0.2223, n=9,8 mice; **$p_{day\ 8}$ = 0.0021, n=11,8 mice). **H.** Representative maximum intensity Z-projections from immunohistochemistry (IHC) of whole-mount mouse skin on Day 2 of the MC903 model. Skin was stained with neuronal marker beta-tubulin III (BTIII; green). Hair follicle autofluorescence is visible in the magenta channel. Images were acquired on a confocal using a 20x water objective. **I.** Quantification of innervation (see Methods) of mouse skin as determined from BTIII staining (*p = 0.012; two-tailed t-test (t =3.114; df =9); n = 7,4 images each from 2 mice per treatment). Day 1 IHC results as follows: 31.78 ± 18.39 % (MC903) and 31.51 ± 16.43 % (EtOH); p = 0.988; two-tailed unpaired t-test; n = 6 images each from 2 mice per treatment.

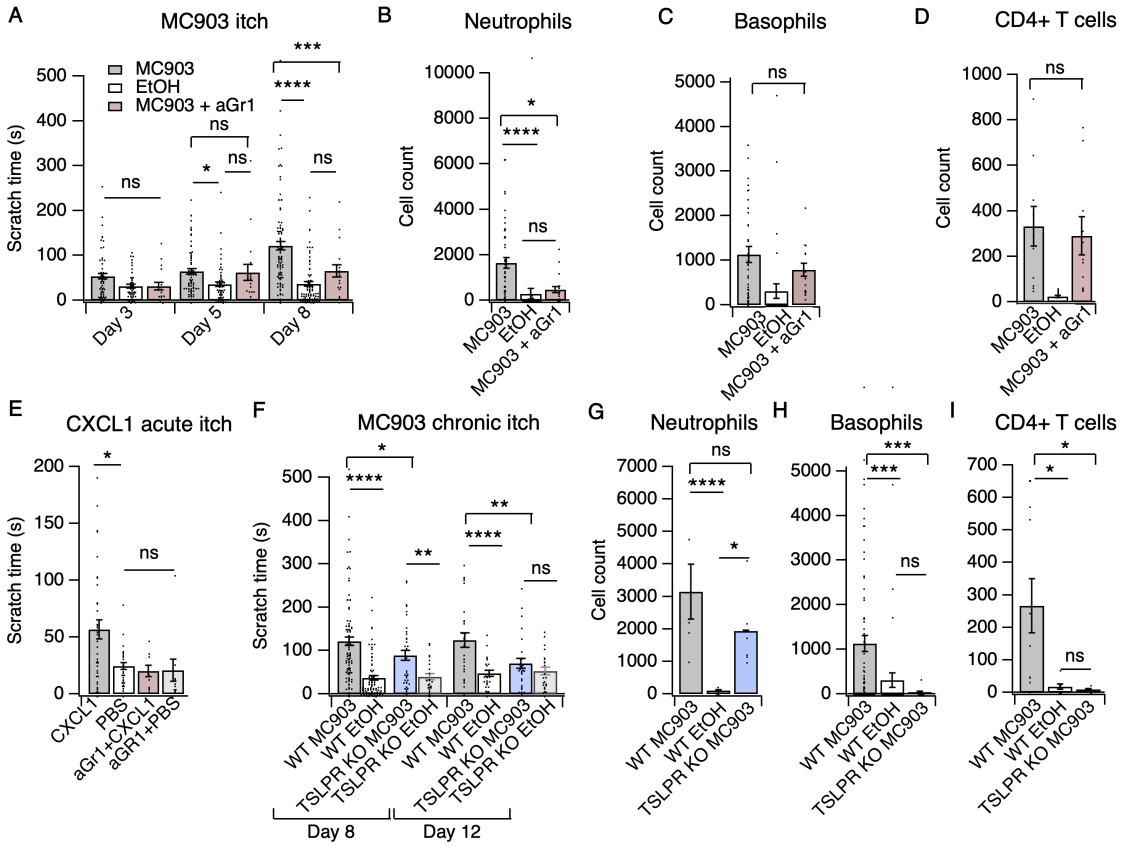


Figure 2. Neutrophils are necessary and sufficient for itch behaviors. A. Scratching behavior of uninjected and PBS-injected mice (combined) and aGr1-injected mice treated with MC903 or ethanol for indicated length of time (two-way ANOVA: ****$p_{interaction} < 0.0001$, F(4,447) = 7.16; Tukey's multiple comparisons: $p_{day\ 3} > 0.05$, n=62,51,17 mice; *$p_{day\ 5} = 0.0154$, $p_{day\ 5} = 0.9854$, $p_{day\ 5} = 0.2267$, n=69,56,17 mice; ****$p_{day\ 8} < 0.0001$, ***$p_{day\ 8} = 0.0007$, $p_{day\ 8} = 0.1543$, n=92,85,17 mice). **B.** Neutrophil count from cheek skin of uninjected/PBS-injected MC903- and ethanol-treated, and aGr1-injected MC903-treated mice on day 8 (one-way ANOVA: ****$p < 0.0001$, F(2,92) = 10.59; Tukey's multiple comparisons: ****$p_{MC903\ vs.\ EtOH} < 0.00001$, n=40,38 mice; $p_{MC903\ vs.\ aGr1\ MC903} = 0.0109$, n=40,17 mice). **C.** Basophil count from cheek skin of uninjected/PBS-injected MC903- and ethanol-treated, and aGr1-injected MC903-treated mice on day 8 (one-way ANOVA: **$p = 0.0025$, F(2,92) = 6.397; Tukey's multiple comparisons: $p_{MC903\ vs.\ aGr1\ MC903} = 0.4848$, n=40,17 mice). **D.** CD4$^+$ T cell count from cheek skin of uninjected/PBS-injected MC903- and ethanol-treated, and aGr1-injected MC903-treated mice on day 8 (two-way ANOVA: *$p_{treatment} = 0.0286$, F(1,26) = 5.373; Holm-Sidak multiple comparisons for PBS versus aGr1: $p_{MC903} = 0.8127$, n=8,10 mice; $p_{EtOH} = 0.7131$, n=6,6 mice). Control MC903 and EtOH data from **Figure 2B-C** are also displayed in **Figure 1**. **E.** Scratching behavior of mice immediately after injection of 1 μg CXCL1 or PBS (i.d. cheek). For neutrophil-depletion experiments, mice received 250 μg anti-Gr1 (aGr1) 20 hours prior to cheek injection of CXCL1 or PBS (one-way ANOVA: ****$p < 0.0001$, F(4,88) = 75.53; Tukey's multiple comparisons: *$p_{CXCL1\ vs.\ PBS} = 0.0126$, n=36,31 mice; $p_{aGr1\text{-}CXCL1\ vs.\ aGr1\text{-}PBS} > 0.9999$, n=10,10 mice; $p_{aGr1\text{-}CXCL1}$ vs. PBS = 0.9986,

n=10,31 mice). **F.** Scratching behavior of WT and TSLPR$^{-/-}$ (TSLPR KO) mice treated with MC903 or ethanol for indicated length of time (two-way ANOVA: ****$p_{interaction}$ < 0.0001, F(9,657) = 4.93; Tukey's multiple comparisons: ****$p_{day\ 8}$ < 0.0001, *$p_{day\ 8}$ = 0.0194, **$p_{day\ 8}$ = 0.0039, n=92,85,36,26 mice; ****$p_{day\ 12}$ < 0.0001, **$p_{day\ 12}$ = 0.0028, $p_{day\ 12}$ = 0.7061, n=26,26,27,23 mice). **G.** Neutrophil count from cheek skin of wild-type MC903- and ethanol-treated, and TSLPR$^{-/-}$ MC903-treated mice on day 5 (two-way ANOVA: **$p_{genotype}$ = 0.0025, F(2,125) = 6.28; Tukey's multiple comparisons: ****$p_{day\ 5\ WT\ MC903\ vs.\ WT\ EtOH}$ < 0.0001, n=6,8 mice; $p_{day\ 5\ WT\ MC903\ vs.\ KO\ MC903}$ = 0.2198, n=6,6 mice; *$p_{day\ 5\ WT\ EtOH\ vs.\ KO\ MC903}$ = 0.0212, n=8,6 mice). **H.** Basophil count from cheek skin of wild-type MC903- and ethanol-treated, and TSLPR$^{-/-}$ MC903-treated mice on day 8 (two-way ANOVA: **$p_{genotype}$ = 0.0023, F(2,117) = 6.38; Tukey's multiple comparisons: ***$p_{day\ 8\ WT\ MC903\ vs.\ WT\ EtOH}$ = 0.0003, n=40,38 mice; ***$p_{day\ 8\ WT\ MC903\ vs.\ KO\ MC903}$ = 0.0003, n=40,15 mice; $p_{day\ 8\ WT\ EtOH\ vs.\ KO\ MC903}$ = 0.5768, n=38,15 mice). **I.** CD4$^+$ T cell count from cheek skin of wild-type MC903- and ethanol-treated, and TSLPR$^{-/-}$ MC903-treated mice on day 8 (one-way ANOVA: **p = 0.0053, F(2,24) = 6.564; Tukey's multiple comparisons: *$p_{WT\ MC903\ vs.\ WT\ EtOH}$ = 0.0163, n=11,8 mice; *$p_{MC903\ vs.\ KO\ MC903}$ = 0.0130, n=11,8 mice; $p_{WT\ EtOH\ vs.\ KO\ MC903}$ = 0.9953, n=8,8 mice). Wild-type MC903 and EtOH data from **2F-H** are also displayed in **Figure 1**.

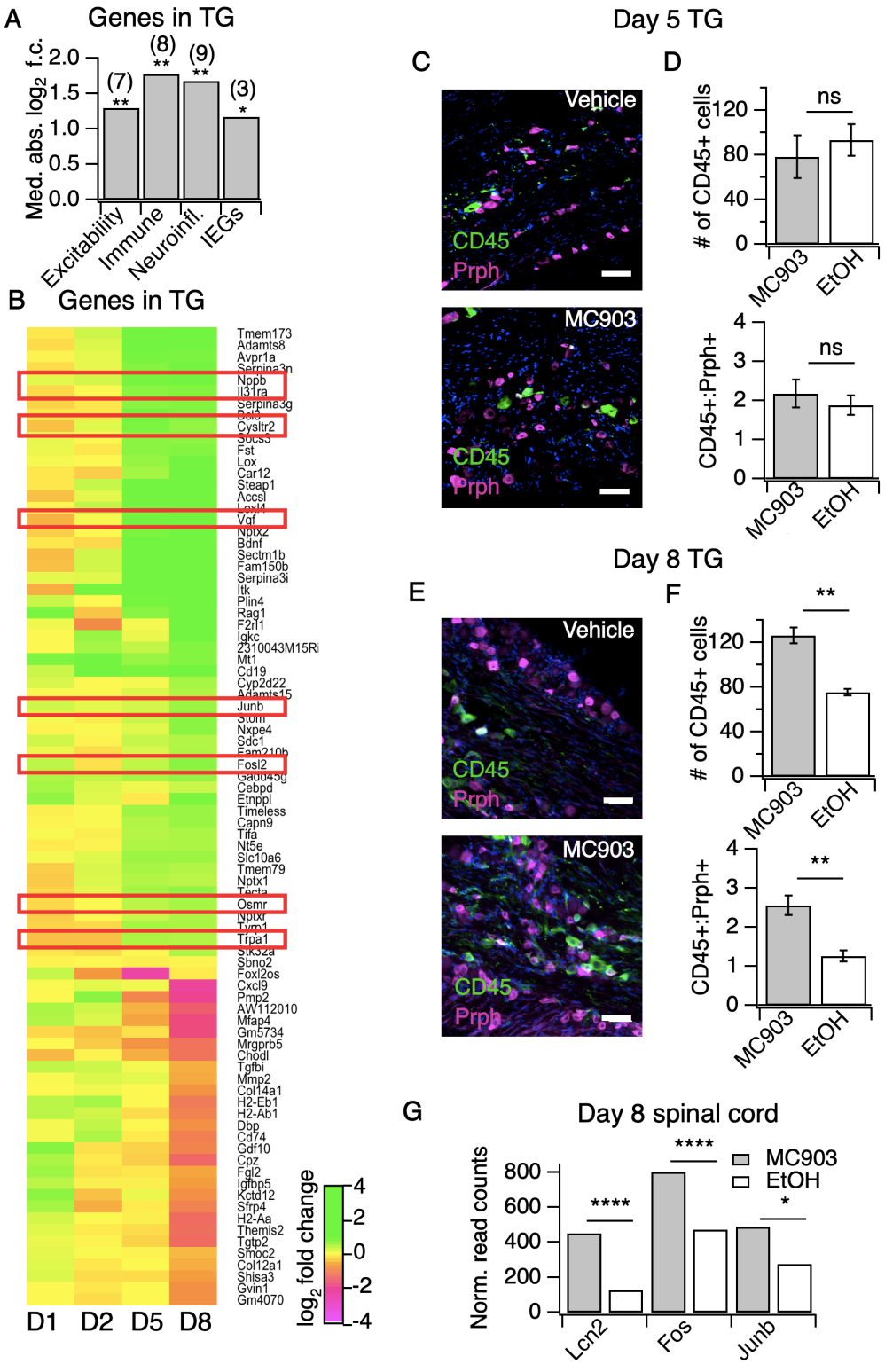


Figure 3. The MC903 model induces rapid and robust changes in neurons. A. Exact permutation test (10,000 iterations, see Methods) for significance of mean absolute $\log_2$ fold change in gene expression at Day 8 (MC903 vs. ethanol) of custom-

defined groups of genes for indicated categories. **B.** Log_2 fold change in gene expression (MC903 vs. ethanol) in mouse trigeminal ganglia (TG) at indicated time points for all genes which were significantly differentially expressed for at least one time point in the MC903 model. Green bars = increased expression in MC903 relative to ethanol; magenta = decreased expression. **C**. Representative composite images showing immune cells (CD45, green), and sensory neurons (Prph, magenta) with DAPI (blue) in sectioned trigeminal ganglia from mice treated with Vehicle or MC903 for five days on the cheek. **D**. Quantification of images examining average number of $CD45^+$ cells per section and average ratio of CD45:Peripherin cells per section after five days of treatment ($p = 0.562$ (t=0.6318, df=4), 0.542 (t=0.6660, df=4); two-tailed unpaired t-tests, n=33-159 fields of view (images) each of both trigeminal ganglia from 3 mice per condition treated bilaterally). **E.** Representative composite images showing immune cells (CD45, green), and sensory neurons (Peripherin (Prph), magenta) with DAPI (blue) in sectioned trigeminal ganglia from mice treated with Vehicle or MC903 for eight days on the cheek. **F**. Quantification of images examining average number of $CD45^+$ cells per section and average ratio of CD45:Peripherin cells per section after eight days of treatment (**$p = 0.0019$ (t=5.977,df=5), **$p = 0.0093$ (t=4.107,df=4); two-tailed unpaired t-tests; n=42-172 fields of view (images) each of both trigeminal ganglia from 3 EtOH or 4 MC903 animals treated bilaterally). Scale bar = 100 μm. Images were acquired on a fluorescence microscope using a 10x air objective. **G.** Log_2 fold change in gene expression (MC903 vs. ethanol) in mouse spinal cord on day 8 showing selected differentially expressed genes ($p_{adjusted} < 0.05$).

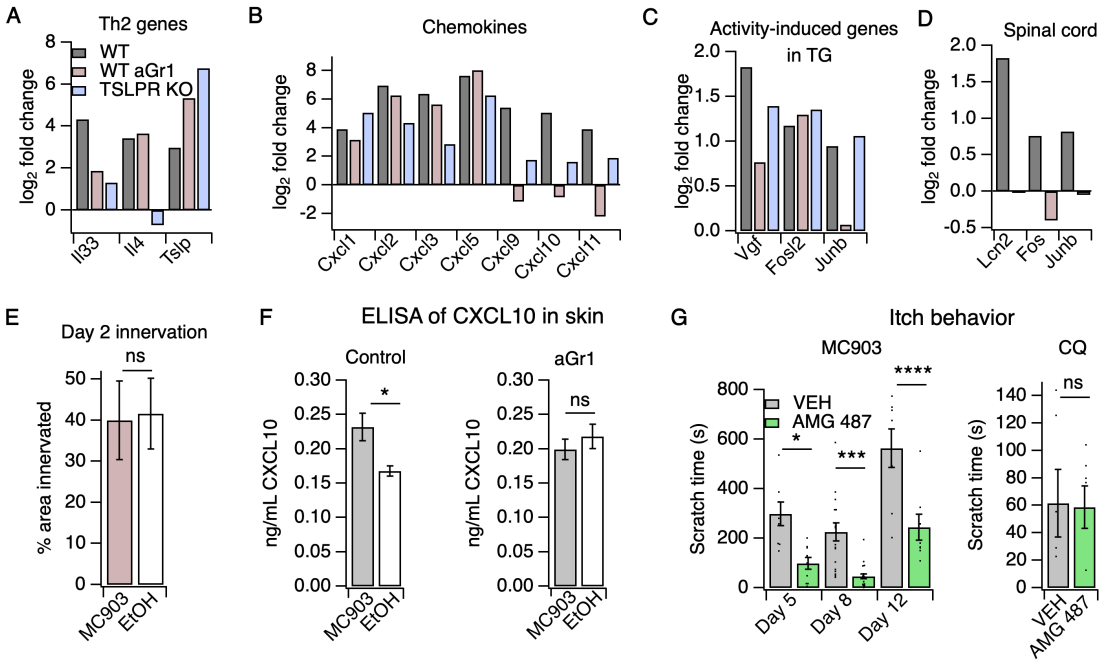


Figure 4. Neutrophils are required for induction of the itch-inducing chemokine CXCL10. A. Log_2 fold change (Day 8 MC903 vs. EtOH) of Th2 genes in skin from uninjected wild-type, aGR1-treated, and $TSLPR^{-/-}$ animals. **B.** Log_2 fold change (Day 8 MC903 vs. EtOH) of chemokine genes in skin from uninjected wild-type, aGr1-treated, and $TSLPR^{-/-}$ animals. **C.** Log_2 fold change (Day 8 MC903 vs. EtOH) of activity-induced genes in trigeminal ganglia from uninjected wild-type, aGr1-treated, and $TSLPR^{-/-}$ animals. **D.** Log_2 fold change (Day 8 MC903 vs. EtOH) of *Lcn2* and activity-induced genes in spinal cord from uninjected and aGr1-treated wild-type mice on day 8. **E.** Quantification of innervation (see Methods) of MC903 and EtOH-treated mouse skin as determined from BTIII staining ($p = 0.8985$; two-tailed t-test ($t = 0.1294$; df =18); n = 9,11 images each from 2 mice per treatment. **F.** CXCL10 levels in skin homogenate as measured by ELISA on day 8 of the MC903 model for uninjected animals (left; $*p = 0.029$ (t=2.715, df=7); two-tailed t-test; n = 4,5 animals) and animals which received aGr1 for 8 days (right; $p = 0.43$ (t=0.815, df=11); two-tailed t-test; n = 6,6 animals). Homogenates were isolated on separate days and so uninjected samples were not compared to aGr1-treated samples. **G.** (Left) Time spent scratching over a thirty minute interval on days 5, 8, and 12 of the MC903 model, one hour after mice were injected with either 3.31 mM of the CXCR3 antagonist AMG 487 or vehicle (10% HPCD; 50 μL s.c. in rostral back); (two-way ANOVA: $****p_{treatment} < 0.0001$, F(1,67) = 50.64; Tukey's multiple comparisons: $*p_{day\ 5} = 0.0216$, n=8,10 mice; $***p_{day\ 8} = 0.0007$, n=18,21 mice; $****p_{day\ 12} < 0.0001$, n=8,8 mice). (Right) Time spent scratching over a thirty minute interval one hour after mice were injected with either 3.31 mM of the CXCR3 antagonist AMG 487 or vehicle (10% HPCD; 50 μL s.c. in rostral back), and immediately after mice were injected with 50 mM chloroquine (20 μL i.d., cheek). p = 0.92 (t=0.0964, df=8); two-tailed t-test; n = 5,5 mice.

A

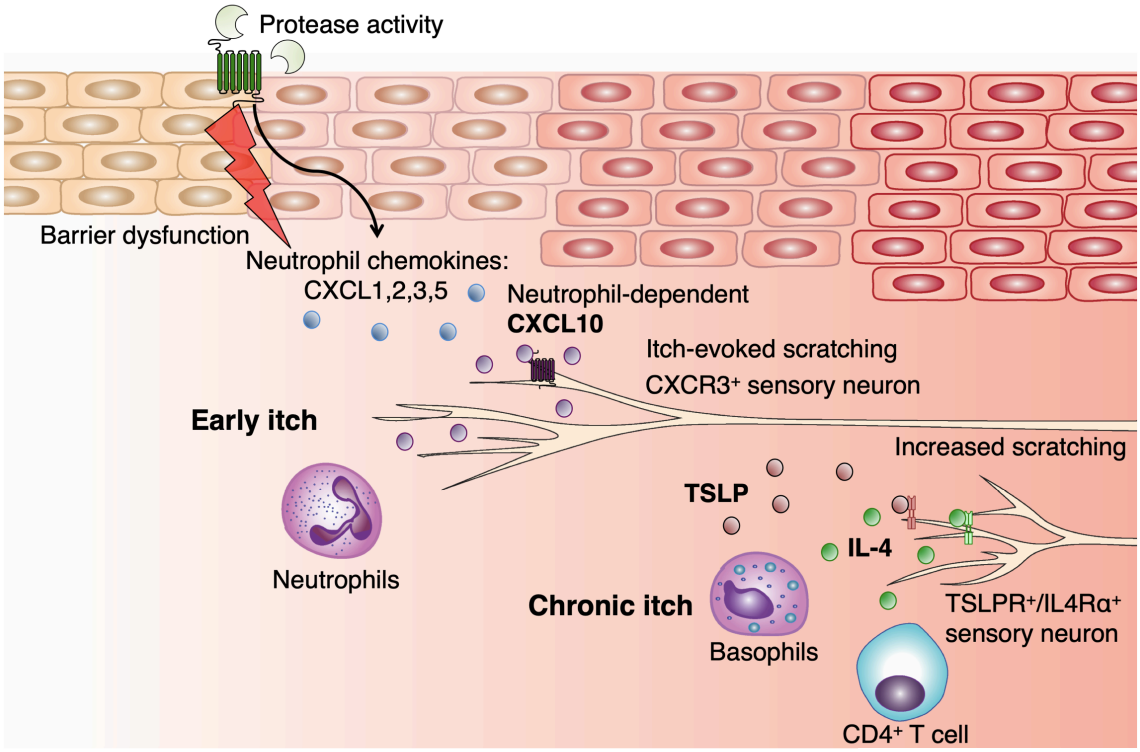


Figure 5. Model of early AD pathogenesis. A. AD induction first results in increased protease expression and barrier dysfunction, which drives production of the cytokines TSLP and CXCL1 via PAR2 activation in keratinocytes. CXCL1 can recruit neutrophils via its receptor CXCR2. Neutrophils may evoke itch by multiple pathways, including degranulation and release of proteases and histamine, production of sensitizing lipids such as PGE_2 and LTB_4, and induction of CXCL10 expression, which can activate sensory neurons via CXCR3. TSLP activates a number of immune cells to elicit IL-4 production, including basophils, which results in increased IL-4, recruitment of $CD4^+$ T cells, and sensitization of neurons to promote itch later in the model (Oetjen et al. 2017).

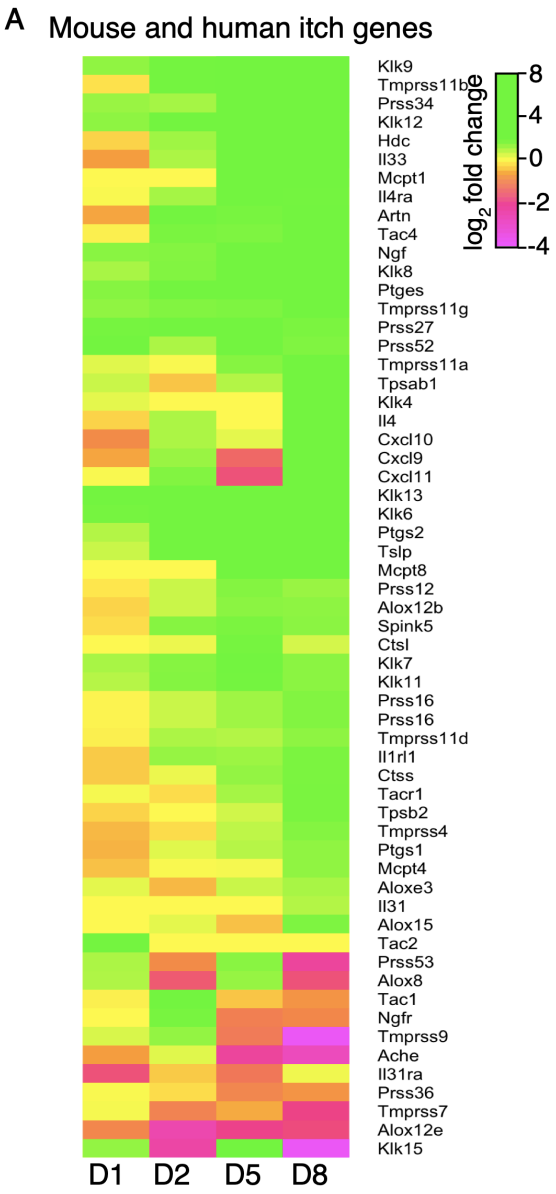


Figure 1-Figure Supplement 1. Expression of mouse and human itch genes. A. Log_2 fold change in gene expression (MC903 vs. ethanol) in mouse skin at indicated time points for genes implicated in mouse or human acute or chronic itch that were significantly differentially expressed for at least one time point in the MC903 model. Green bars = increased expression in MC903 relative to ethanol; magenta = decreased expression.

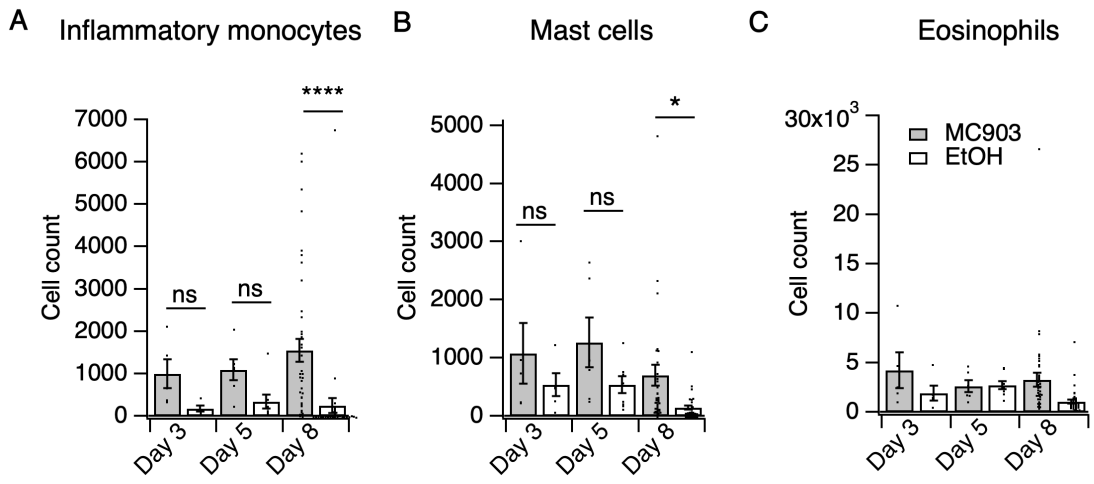


Figure 1-Figure Supplement 2. Immune cell counts in MC903-treated skin. A. Inflammatory monocyte counts in MC903- and ethanol-treated skin at indicated time points (two-way ANOVA: *$p_{treatment}$= 0.0129, F(1,96) = 6.42; Sidak's multiple comparisons: $p_{day\ 3}$ = 0.6933, n=5,5 mice; $p_{day\ 5}$ = 0.6536, n=6,8 mice; ****$p_{day\ 8}$ < 0.0001, n=40,38 mice). **B.** Mast cell counts in MC903- and ethanol-treated skin at indicated time points (two-way ANOVA: *$p_{treatment}$= 0.0117, F(1,96) = 6.60; Sidak's multiple comparisons: $p_{day\ 3}$ = 0.6562, n=5,5 mice; $p_{day\ 5}$ = 0.2829, n=6,8 mice; *$p_{day\ 8}$ = 0.0115, n=40,38 mice). Data from mice receiving i.p. injection of PBS (see Figure 4) in addition to MC903 or EtOH are also included. **C.** Eosinophil counts in MC903- and ethanol-treated skin at indicated time points (two-way ANOVA: $p_{treatment}$= 0.0964, F(1,96) = 2.81, n=4,5,5,8,38,38 mice).

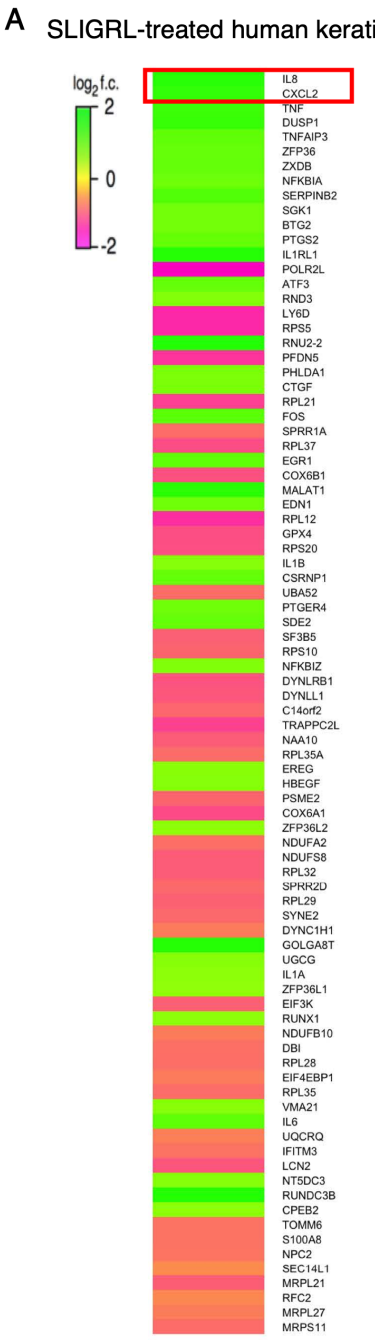


Figure 1-Figure Supplement 3. Protease receptor activation triggers rapid upregulation of neutrophil chemokine genes in human keratinocytes. A. Heat map showing $\log_2$ fold change in gene expression in cultured human keratinocytes 3 hours after SLIGRL treatment (100 μM; bottom; see **Figure 1-source data table 7**) compared to vehicle controls, as measured by RNA-seq. Genes are sorted by descending corrected *p*-value; only significantly differentially expressed ($p < 0.05$) are displayed.

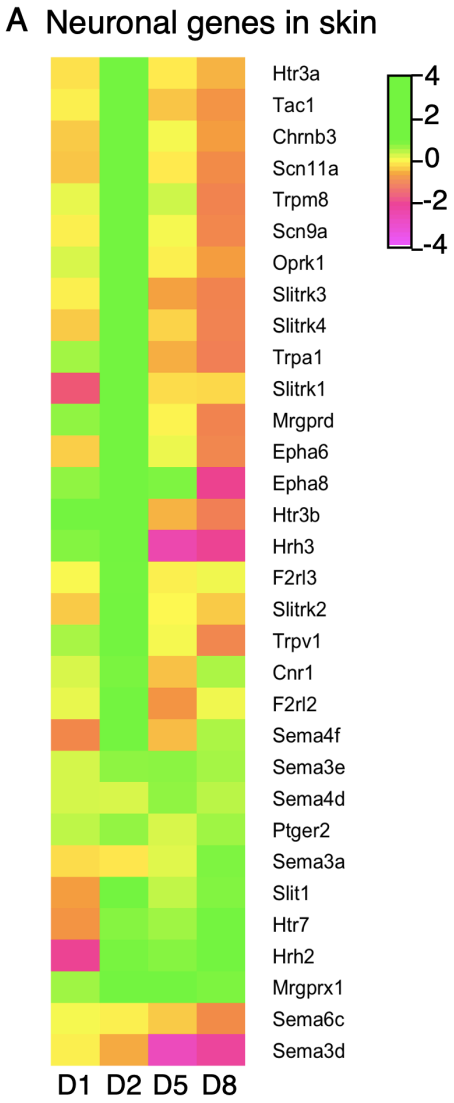


Figure 1-Figure Supplement 4. Expression of neuronal genes and axon guidance molecules in skin. A. Log_2 fold change in gene expression (MC903 vs. EtOH) in mouse skin at indicated time points for markers of locally translated sensory neuronal transcripts or genes implicated in neurite remodeling and/or axon guidance that were significantly differentially expressed for at least one time point in the MC903 model. Green bars = increased expression in MC903 relative to ethanol; magenta = decreased expression.

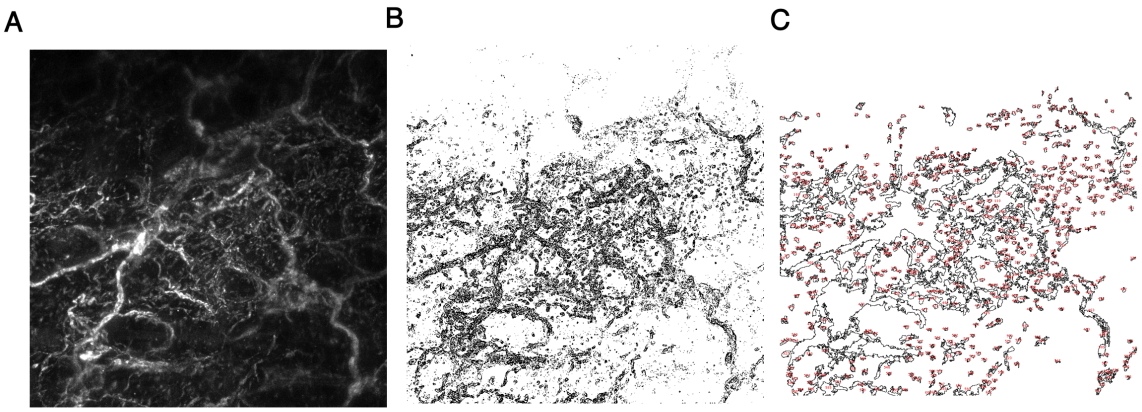


Figure 1-Figure Supplement 5. Method of image quantification for whole mount skin. **A.** Representative maximum intensity z-projection of beta tubulin III staining in cheek skin. **B.** Binary image after edge-detection. **C.** % Area innervated was calculated from the percentage of the image area which was occupied by the regions of interest (ROIs) outlined in red.

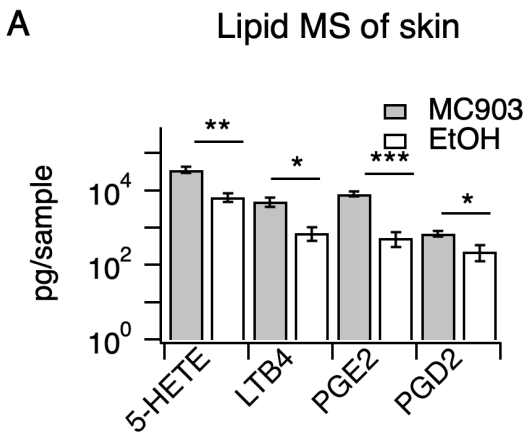


Figure 1-Figure Supplement 6. Inflammatory lipids in MC903-treated skin. A. Quantification of indicated lipids from 6 mm biopsy punches of cheek skin of MC903- and EtOH-treated mice (at day 8) by LC-MS/MS (**p = 0.006 (*t*=4.148,*df*=6), *p = 0.024 (*t*=3.003,*df*=6), ***p = 0.0007 (*t*=6.392,*df*=6), *p = 0.022 (*t*=3.058,*df*=6); two-tailed unpaired t-tests; n = 4 mice per group).

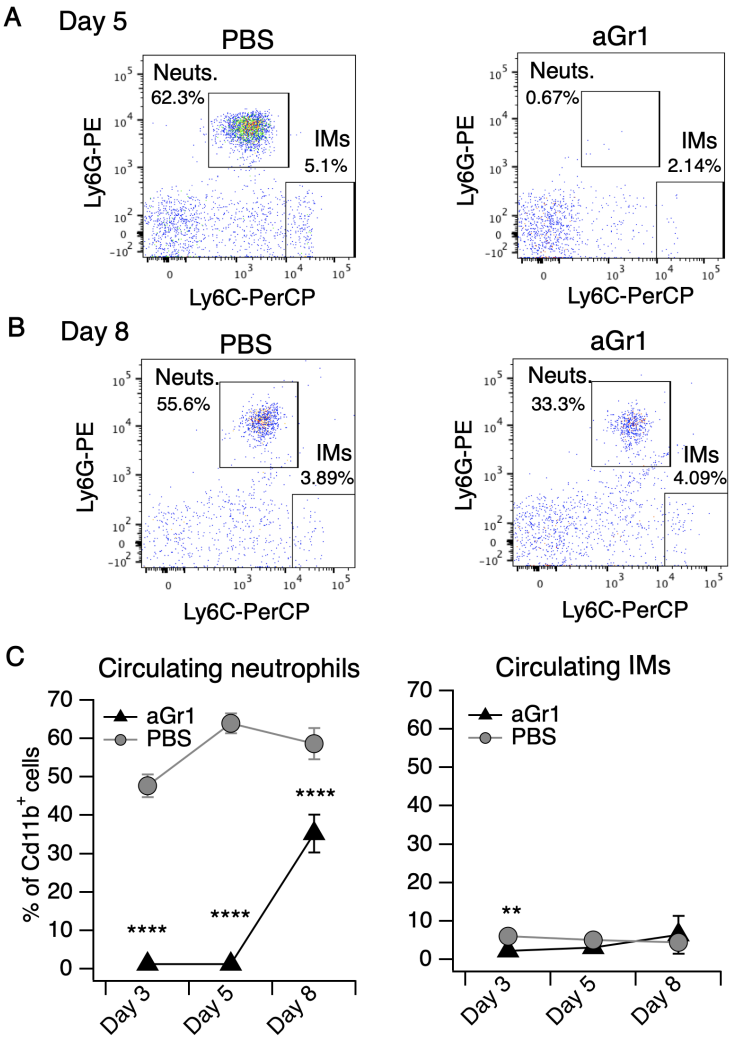


Figure 2-Figure Supplement 1. aGr1 treatment preferentially depletes neutrophils. **A.** Representative flow cytometry plots of cells collected from blood of mice injected with PBS or aGr1 (250 µg, i.p.) once-daily for five days concurrent with daily MC903 topical treatment. Shown are CD45.2^{+}CD11b^{+} cells, plotted by Ly6G and Ly6C signal, with neutrophil (Neuts.) and inflammatory monocyte (IMs) populations indicated. Neutrophils were defined as Cd11b^{+}Ly6G^{+}Ly6C$^{mid/high}$ and IMs were defined as Cd11b^{+}Ly6G^{-}Ly6C^{high} (see Methods). **B.** Representative flow cytometry plot as in **A**, depicting neutrophil and IM populations from blood collected on day 8. **C. (Left)** Neutrophil counts in blood shown as % of Cd11b^{+} cells from aGr1/MC903 (black triangles) and PBS/MC903 (gray circles)-treated animals on days 3, 5, and 8 of the model (two-way repeated measures ANOVA: ****$p_{treatment} < 0.0001$, F(1,31) = 299.5; Sidak's multiple comparisons: ****$p_{day\ 3} < 0.0001$; ****$p_{day\ 5} < 0.0001$; ****$p_{day\ 8} < 0.0001$, n= 16,17 mice). **(Right)** Inflammatory monocyte counts in blood shown as % of Cd11b^{+} cells from aGr1/MC903 and PBS/MC903-treated animals on days 3, 5, and 8 of the model (two-

way repeated measures ANOVA: *$p_{\text{treatment}}$ = 0.0468, F(1,31) = 4.287; Sidak's multiple comparisons: **$p_{day\ 3}$ = 0.0015; $p_{day\ 5}$ = 0.1918; $p_{day\ 8}$ = 0.2013, n= 16,17 mice).

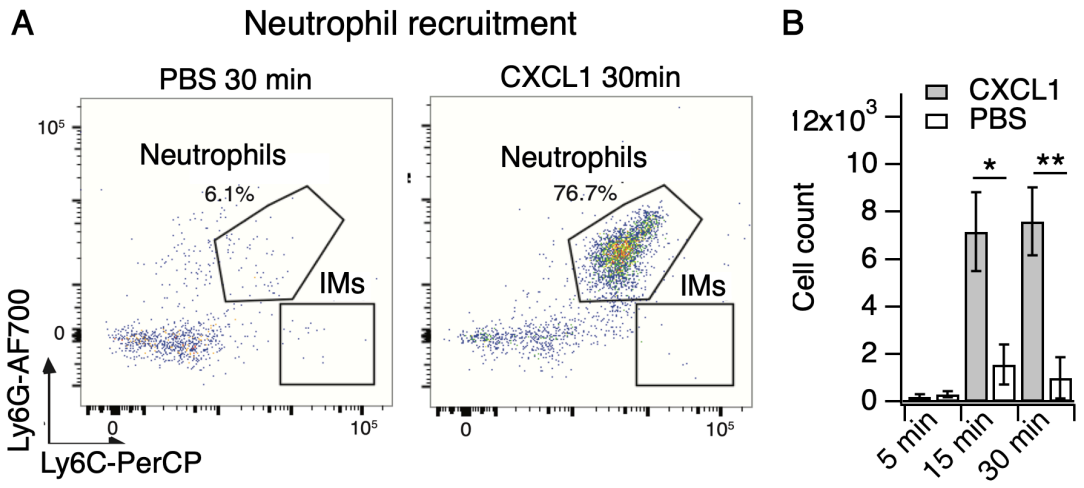


Figure 2-Figure Supplement 2. CXCL1 rapidly and selectively recruits neutrophils to skin. A. Representative flow cytometry plots of cells from cheek skin of mice injected with PBS or CXCL1 (1 µg, i.d.). Shown are CD45.2^{+}CD11b^{+} cells, plotted by Ly6G and Ly6C signal, with neutrophil and inflammatory monocyte (IM) populations indicated. **B.** Neutrophil count from cheek skin of mice 5, 15, and 30 minutes after injection of CXCL1 or PBS (two-way ANOVA: *$p_{\text{interaction}}$= 0.0239, F(2,21) = 4.48; Sidak's multiple comparisons: $p_{5\ min}$ > 0.9999, n=4,5 mice; *$p_{day\ 15\ min}$ = 0.0141, n=4,4 mice; **$p_{day\ 30\ min}$ = 0.0031, n=3,7 mice).

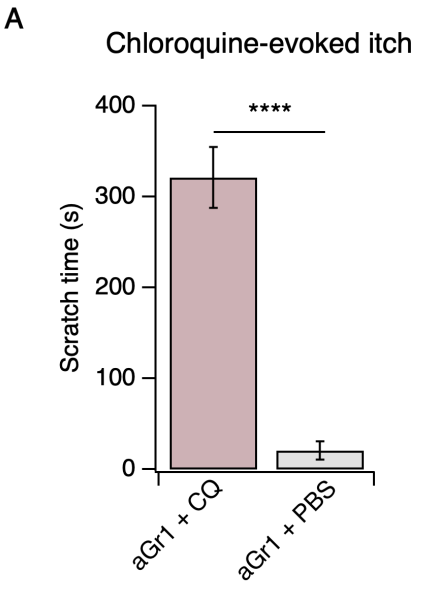


Figure 2-Figure Supplement 3. Neutrophil depletion does not affect chloroquine-evoked itch. A. Scratching behavior of mice immediately after injection of chloroquine (CQ) or PBS (i.d. cheek). For neutrophil-depletion experiments, mice received 250 μg anti-Gr1 (aGr1) 20 hours prior to cheek injection of CQ or PBS (two-tailed t-test: ****$p <$ 0.0001 (t=10.58, df=14); n=6,10 mice).

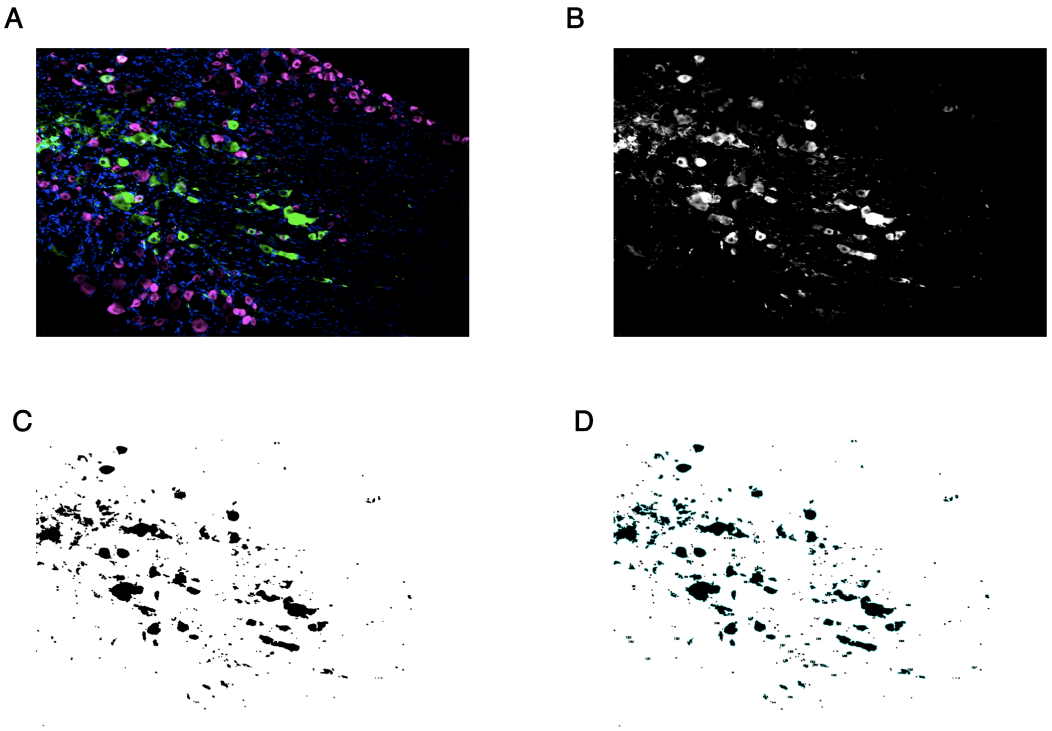
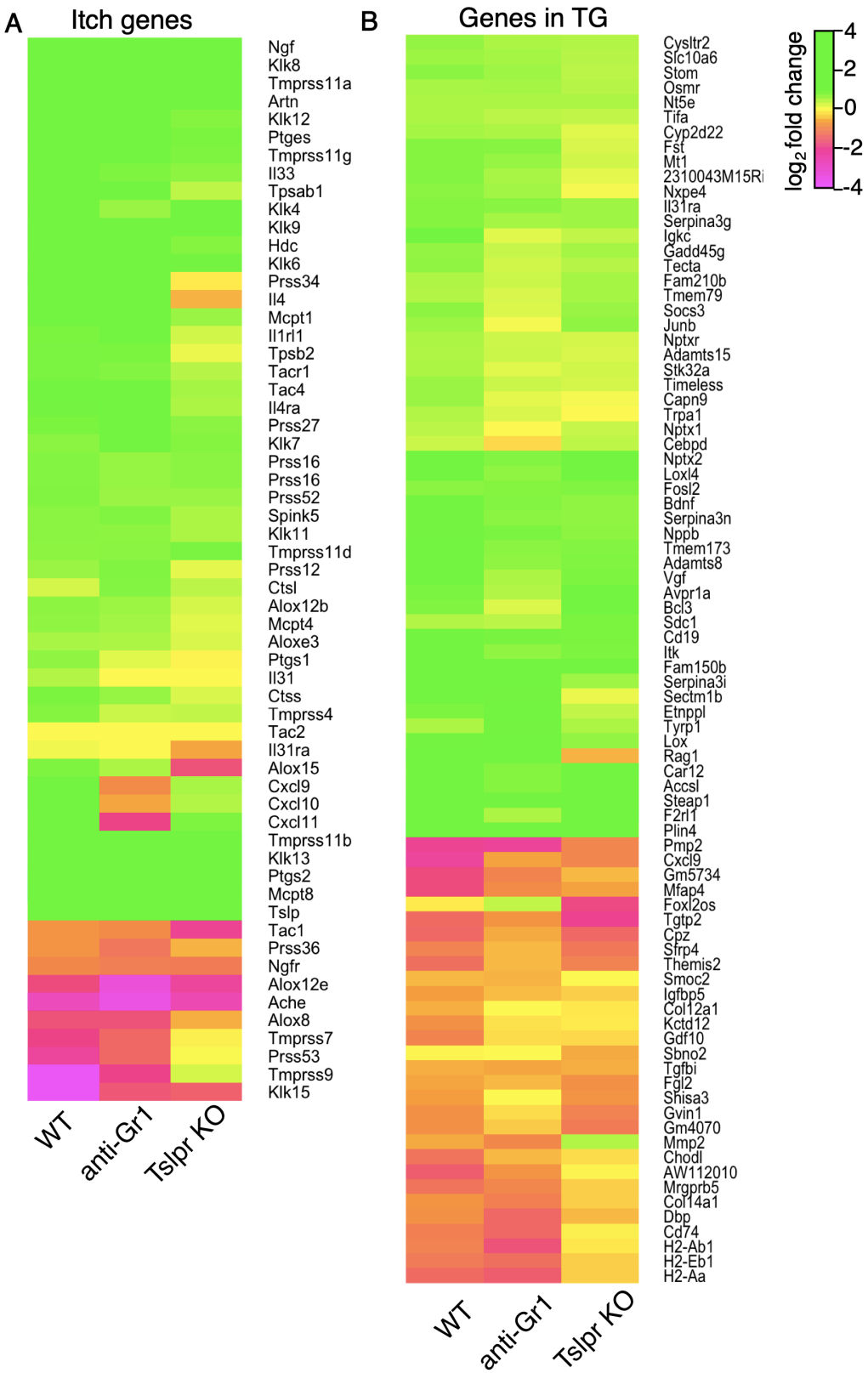


Figure 3-Figure Supplement 1. Method of image quantification for sectioned trigeminal ganglia. **A.** Representative composite image showing CD45 (green), Peripherin (magenta), and DAPI (blue). **B.** Single-channel CD45 image with automated min/max intensity thresholding. **C.** Resultant binary image generated from **B. D.** Cells were counted as the number of regions of interest (ROIs) outlined in blue.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Itch genes
Ngf
Klk8
Tmprss11a
Artn
Klk12
Ptges
Tmprss11g
Il33
Tpsab1
Klk4
Klk9
Hdc
Klk6
Prss34
Il4
Mcpt1
Il1rl1
Tpsb2
Tacr1
Tac4
Il4ra
Prss27
Klk7
Prss16
Prss16
Prss52
Spink5
Klk11
Tmprss11d
Prss12
Ctsl
Alox12b
Mcpt4
Aloxe3
Ptgs1
Il31
Ctss
Tmprss4
Tac2
Il31ra
Alox15
Cxcl9
Cxcl10
Cxcl11
Tmprss11b
Klk13
Ptgs2
Mcpt8
Tslp
Tac1
Prss36
Ngfr
Alox12e
Ache
Alox8
Tmprss7
Prss53
Tmprss9
Klk15
WT
anti-Gr1
Tslpr KO
B
Genes in TG
Cysltr2
Slc10a6
Stom
Osmr
Nt5e
Tifa
Cyp2d22
Fst
Mt1
2310043M15Ri
Nxpe4
Il31ra
Serpina3g
Igkc
Gadd45g
Tecta
Fam210b
Tmem79
Socs3
Junb
Nptxr
Adamts15
Stk32a
Timeless
Capn9
Trpa1
Nptx1
Cebpd
Nptx2
Loxl4
Fosl2
Bdnf
Serpina3n
Nppb
Tmem173
Adamts8
Vgf
Avpr1a
Bcl3
Sdc1
Cd19
Itk
Fam150b
Serpina3i
Sectm1b
Etnppl
Tyrp1
Lox
Rag1
Car12
Accsl
Steap1
F2rl1
Plin4
Pmp2
Cxcl9
Gm5734
Mfap4
Foxl2os
Tgtp2
Cpz
Sfrp4
Themis2
Smoc2
Igfbp5
Col12a1
Kctd12
Gdf10
Sbno2
Tgfbi
Fgl2
Shisa3
Gvin1
Gm4070
Mmp2
Chodl
AW112010
Mrgprb5
Col14a1
Dbp
Cd74
H2-Ab1
H2-Eb1
H2-Aa
WT
anti-Gr1
Tslpr KO
log2 fold change
4
2
0
-2
-4

Figure 4-Figure Supplement 1. MC903-dependent gene expression changes in aGr1-treated and TSLPR$^{-/-}$ animals. A. Heat map showing log_2 fold change in gene expression (Day 8 MC903 vs. EtOH) for itch-associated genes in wild-type, aGr1-treated, and TSLPR$^{-/-}$ skin. Green bars = increased expression in MC903 relative to ethanol; magenta = decreased expression. **B.** Heat map showing log_2 fold change in gene expression (Day 8 MC903 vs. EtOH) for wild-type, aGr1-treated, and TSLPR$^{-/-}$ mouse trigeminal ganglia (TG) at indicated time points for all genes which were significantly differentially expressed for at least one time point in the MC903 model (See **Figure 2D**). Green bars = increased expression in MC903 relative to ethanol; magenta = decreased expression.

Chapter 3: Future Perspectives.

Although I was fortunate enough to work on multiple projects throughout my time as a PhD student at Berkeley, the central focus of my thesis was atopic dermatitis pathogenesis. This research continued to evolve and change throughout the time that I was involved in the project, and in fact our data led us in many directions that we did not initially expect. In addition, this work was highly collaborative, and multiple authors contributed unique ideas, expertise, and experience, each of which added a new dimension to the paper. For these reasons, it is easy to imagine many further lines of investigation that could be followed in the future.

One potential avenue for future research is the role of leukotriene B_4 (LTB_4) in chronic itch, and particularly in the MC903 model of atopic dermatitis. LTB_4 is a proinflammatory lipid known to play a role in neutrophil swarming (Lämmermann et al., 2013). Increased levels of LTB_4 have been observed in the lesional skin of atopic dermatitis patients (Fogh et al., 1989), and LTB_4 has been shown to trigger acute scratching behaviors (Andoh and Kuraishi, 1998). Studies also indicate that application of LTB_4 to cultured somatosensory neurons isolated from the dorsal root ganglia evokes increased intracellular calcium (Andoh and Kuraishi, 2005). I was able to use LTB_4 application to evoke calcium transients in cultured somatosensory neurons from the dorsal root ganglia, but intriguingly, I needed to use a very high concentration of LTB_4 (100 uM) to achieve these results. In contrast, LTB_4 evoked increases in intracellular calcium levels in cultured neutrophils from bone marrow at nanomolar levels. Although BLT1 (the high-affinity LTB_4 receptor via which LTB_4 is believed to exert its effects on neurons) RNA has been detected at low levels in somatosensory neurons (Usoskin et al., 2015), some work suggests that it exerts its effects on neurons by functioning as a weak activator of TRPV1 rather than by acting directly on BLT1 (Hwang et al., 2000). It would be interesting to culture somatosensory neurons from the dorsal root ganglia of BLT1 and TRPV1 knock-out mice and observe the calcium transients elicited by application of LTB_4. If the calcium transients are abolished in TPRV1, but not BLT1, knock-out mice, this would support the hypothesis that the calcium transients that I observed are a result of LTB_4 is acting upon neuronal TRPV1. A role for TRPV1 in prostaglandin signaling would be fascinating, and could have implications for the development of pain management therapeutics. Our work with LTB_4 in cultured neurons led us to examine the BLT1 knock-out animals using the MC903 mouse model of atopic dermatitis. We found that these mice had a deficit in scratching behaviors at day 5, but scratched normally at day 8. These results were somewhat puzzling, and since the deficit was subtle, we eventually moved on to other mechanisms by which neutrophils could drive itch behaviors. What role, if any, LTB_4/BLT1 signaling plays in atopic dermatitis remains enigmatic.

Another interesting and quite open question has to do with the mechanisms by which thymic stromal lympopoietin (TSLP) exerts its effects in atopic dermatitis. TSLP is sometimes called a “master regulator” of allergic inflammation and is known to bias the inflammatory response towards a Th2 type polarization (Liu, 2006). This cytokine signals through a heterodimeric receptor composed of the IL7Rα chain and the TSLPR

chain, which is unique to the TSLP response. A wide variety of immune cells, including T cells, dendritic cells, B cells, basophils, mast cells, and neutrophils, express the TSLP receptor, TSLPR (He and Geha, 2010). Our project's focus on neutrophils has made the role of TSLPR signaling in these cells particularly interesting to me. A study from West et al. found that TSLPR signaling in neutrophils increased production of reactive oxygen species in the context of *Staphylococcus aureus* infection (West et al., 2016). Furthermore, TSLPR is expressed in somatosensory neurons, and application of TSLP to cultured dorsal root ganglia somatosensory neurons evokes calcium transients (Oetjen et al., 2017; Wilson et al., 2013) and sensitizes cultured neurons such that they display increased responses to known pruritogens (Oetjen et al., 2017). Intriguingly, it also evokes acute scratching behaviors in mice upon injection into the cheek (Wilson et al., 2013). Taken together, these studies suggest that TSLP may play a variety of roles in atopic dermatitis pathogenesis via actions on diverse cell types.

By using a TSLPR knockout mouse and the MC903 mouse model of atopic dermatitis, we found that TSLPR signaling was required for itch behaviors at day 12 of the treatment. However, we were surprised to discover that the mice still scratched robustly at day 8, suggesting that TSLPR signaling does not drive itch at this earlier timepoint. We further examined immune cell infiltration into the and found deficits in T cell and basophil recruitment, which (a) dovetails nicely with previous work showing that TSLPR signaling is required for recruitment of these cell types and (b) supports the conclusion that T cells and basophils are not required for itch during the early development of atopic dermatitis. An interesting avenue of further research would be to repeat these experiments using conditional knockout animals. Considering previously published work highlighting a role for TSLPR signaling in neurons and neutrophils and our own finding that neutrophils are essential for itch behaviors in atopic dermatitis, it would be particularly interesting to examine mice lacking the TSLPR receptor specifically in somatosensory neurons and neutrophils in order to determine which cell types are required for the deficit in scratching behaviors. Reactive oxygen species are known to evoke itch (Liu and Ji, 2012), and since West et. al. found that TSLPR signaling in neutrophils increased release of reactive oxygen species (ROS), it is possible that altered production of ROS contributes to the itch deficit observed in the TSLPR knockout animals.

Indeed, we observed a deficit in scratching behaviors at day 12 in both animals lacking TSLPR and in animals injected with a CXCR3 antagonist. We originally hypothesized that the CXCR3/neutrophil signaling pathway and the TSLPR signaling pathways were separate, with neutrophils/CXCR3 signaling being required for itch in the first week of the MC903 model, whereupon TSLPR signaling and the cells of the adaptive immune system, particularly CD4+ Th2 cells, would start to drive scratching behaviors. However, our observation that CXCR3 blockade at day 12 of the model also ablates itch behaviors suggests that the story is more complex. There may be crossover between these two pathways that we do not yet fully understand: perhaps the TSLPR pathway drives itch at later timepoints in part by stimulating the release of cytokines from immune cells and/or neurons that then evoke CXCL10 release from keratinocytes.

Our neutrophil depletion consistently ablated itch behaviors at day 8 of the MC903 model, but the precise details of the mechanism by which neutrophils drive itch has remained somewhat elusive. Our data suggest that CXCL10 signaling via the CXCR3 receptor is required for itch behaviors, and we have shown by ELISA that neutrophil depletion with aGr1 ablates the increased production of CXCL10 normally observed in the skin of MC903 treated animals. However, the link between neutrophil recruitment and CXCL10 production is unclear. Keratinocytes, myeloid cells and sensory neurons have all been shown to produce CXCL10 (Flier et al., 2001; Hashimoto et al., 2018; Ioannidis et al., 2016; Kanda et al., 2007; Koga et al., 2008; Michalec et al., 2002; Padovan et al., 2002; Steain et al., 2011; Tamassia et al., 2007). Do neutrophils drive the release of CXCL10 from keratinocytes? If so, how? The subtle phenotype that we observed in the BLT1 KO animals, mentioned earlier in this chapter, has led me to believe that LTB4/BLT1 signaling could be involved in driving CXCL10 release from keratinocytes. The lack of a more dramatic phenotype in these animals, though, suggests that other pathways are also important. It would be interesting to measure CXCL10 release using an ELISA in the BLT1 KO animals at days 5 and 8 of MC903 treatment to determine whether or not CXCL10 production is impaired in these mice. A particularly fascinating outcome would be impaired CXCL10 production at day 5, but not day 8, of MC903 treatment, which would mirror the subtle itch deficit that we observed. An important caveat with this experiment is that while expression of CXCL10 as measured by RNA Seq was slightly increased at day 5, it was not statistically significant. This would likely make ascertaining differences in CXCL10 protein at day 5 of MC903 treatment between wild-type and BLT1 KO mice challenging.

Finally, I have been curious for a couple of years now as to what the CD4+ T cell polarization looks like in the context of the MC903 atopic dermatitis model. This is an aspect of the model that we have discussed frequently in the lab, but I have not been able to conduct the necessary experiments to address the question due to time constraints. It was long believed that atopic dermatitis was a strongly Th2 polarized disease, and while this is the case for many patients, the story has turned out to be quite a bit more nuanced. Research has now shown that many patients exhibit a mixed Th1/Th2 polarization, with some patients additionally having contributions from Th17 and/or Th22 cells. These differences seem to stem partly from differences in age and ethnic background: young patients and Asian patients tend to exhibit more of a Th17 type response than do adult European patients (Guttman-Yassky and Krueger, 2017). With this in mind, I am particularly interested to know what the polarization of the CD4+ T cells in the MC903 model is, because it would give us a sense of what ‘type’ of atopic dermatitis we are modeling. I also suspect that this polarization might change in the neutrophil depleted animals: I found that CD4+ T cells still infiltrate the skin at days 5 and 8 of MC903 treatment in mice lacking neutrophils, but was not able to investigate their polarization. A very small amount of preliminary data suggests that depletion of neutrophils on days 0 – 7 of treatment ablates itch behaviors at day 12, even though neutrophils begin to rebound in the blood by day 8 of our depletion protocol. If these preliminary data were repeated, they would support the hypothesis that the neutrophils may be required early on in the model to ‘set up’ the chronic inflammation that drives

scratching in the second week of the model, and I wonder if this could be related to CD4+ T cell polarization.

The questions I raise in the preceding pages outline just a few of the potential experiments that could conceivably be pursued as a follow-up to the work that my co-authors and I completed. It has been a privilege to be part of such a complex and far-reaching project and to do research alongside an incredibly talented group of people. I am deeply grateful to everyone involved.

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Chapter 1

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